

FERMENTED FAVA BEAN-BASED DIETS & DOG CARDIAC HEALTH: DOES PROTEIN
CONTENT OR TAURINE MATTER?

A Thesis Submitted to the
College of Graduate and Postdoctoral Studies
In Partial Fulfillment of the Requirements
For the Degree of Master of Science
In the Department of Veterinary Biomedical Sciences
University of Saskatchewan
Saskatoon

By

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ABSTRACT

The pet food industry has continued major growth for several decades, leading to novel ingredients gaining attention that requires scientific investigation. Fava bean is a pulse largely produced in Canada that, although not yet approved as a pet food ingredient, is a great source of protein, fiber, and starch. Various pulses and legumes are already being used to replace grains in dog diet composition with few issues. Nevertheless, in 2018 the Food and Drugs Administration stated that grain-free diets were linked to dilated cardiomyopathy (DCM) in dogs, but no robust evidence to date has proven this connection. Despite desirable agronomic and nutritional qualities, the fava bean has a fairly high concentration of anti-nutritional factors. In particular, vicine and convicine result in favism in susceptible humans which causes acute hemolytic anemia. Such a condition, however, has not been described in dogs. When processed through cooking, extrusion or fermentation, pulses and legumes can have anti-nutritional factors altered and most often reduced.

The purpose of this study was to analyze the effects of yeast fermentation of two varieties of fava beans (low or high tannin variety), when used as a pet food ingredient, on dogs' overall and cardiovascular health as well as nutrient digestibility. Moreover, two commercial diets, grain-free or grain-containing, with different protein levels (24.6% crude protein (CP) on dry matter (DM) basis versus 41.4% CP (DM) on the first study; 31.3% CP (DM) versus 41.4% CP (DM) on the second study), were used as a comparison and the same parameters were evaluated. The first study was a short-term period of seven days of feeding each diet. By the end of each period, blood analyses, cardiovascular testing, and digestibility protocols were performed. As a result, fava bean-based diets did not cause hemolytic anemia in dogs or cause adverse changes in blood chemistry or counts. Thus, fava bean was shown to be safe as a dog food ingredient, at least in the short term. In contrast, after seven days of feeding, the normal protein level, grain-containing commercial diet appeared to cause some cardiovascular alteration that could lead to undesirable cardiac changes in the long term. Also, fermentation of the fava bean flour with *Candida utilis* prior to use in dog foods presented positive results as a bioprocessing technique that reduced anti-nutritional factors. A 28-day feeding period was examined in the second study and analyzed the same parameters as the first study, with the addition of cardiovascular biomarkers and bile salt content in the feces. The results from the second study demonstrated that even after a longer period, fava bean-based diets did not cause adverse cardiac or vascular

alterations. The beagles fed both of the commercial diets and all fava bean-based diets did not show any tendency to develop DCM. Moreover, the high protein, grain-free, commercial diet appeared instead to show improved cardiovascular functioning. Also, fermentation increased energy, nutrient digestibility and red blood cells which are positive effects of this bioprocessing technique. On the other hand, these benefits of fava bean-based diets were counteracted by impaired glucose handling. In conclusion, this thesis shows that yeast fermentation of fava bean flour prior to use in dog foods led to improved health parameters. Also, fava bean appears to be a safe pet food ingredient. Moreover, the commercial grain-free, high protein diet did not cause adverse effects on cardiovascular performance in the resistant beagle breed after a 7 or 28-day period.

ACKNOWLEDGMENTS

I would like to thank you, Dr. Lynn Weber, for being an excellent, attentive, and supportive supervisor. The opportunity and guidance you gave me from the beginning were pivotal keys that made me grow as a critical thinker and in the science field. For all the trust in my work, I thank you.

I thank my parents, Antônio Henrique and Rita, and sisters, Ana Cláudia and Flávia, for giving me the support and love I needed, even from a long distance. I hope I made all of you proud.

Special thanks to my husband and best friend, Lucas, without whom I wouldn't get here. You were there when I most needed it with the greatest positive words, arms wide open to hold me when I thought I couldn't make it, and lots of love and laughs to relieve the stress. Thanks for helping me with your expertise in academic work and statistical analysis. Heitor, thank you for being so patient with me and for showing me so much love even when I thought I wasn't there enough for you. You were and will always be my booster, guiding me on trying to be my best version.

Thank you, Tressa Morris, for being my first friend and work partner in this journey. I couldn't ask for a better person to share the fermentation days with. You turned the challenging first months into smooth sailing. Thanks for sticking around with me even after that, for being a helpful hand with the dogs, and for your friendship. You will be an amazing vet!

I would like to thank you, Priscila Curso, for sharing your experiences, teaching the fermentation steps and for your friendship.

Thank you, Chloe Quilliam, for your help with the dogs and work. Thanks for the partnership when we shared classes and the same phases in this exciting graduate student life.

Thank you, Cindy Pollard, for being efficient and proactive in helping all the VBMS grad students. Thanks to all VBMS and WCVVM staff that make the building a pleasant place to work.

Thank you all, Weber lab team. I wish COVID weren't here so we could spend more time together. Anyways, it was great to share with you ideas and presentations in our in-person and online meetings.

Thank you to my committee members, Dr. Matthew Loewen and Dr. Suraj Unniappan, for the guidance and suggestions to make this work possible.

Thank you to all the Animal Care Unit staff for taking good care of the beagles and helping in many ways during the trial days. Thanks to the Canadian Feed Research Centre for the assistance in the diet preparation.

Thank you, Milka, Twix, Hershey, Joy, Roca, Crunch, Chip, Henry and Reese, for allowing me to show love and work with you and teaching me to be patient. Also, many thanks to all the volunteers who were there to play, show love, and walk the dogs.

I would like to thank the funding agencies Saskatchewan Pulse Growers, Western Grains Research Foundation, and the Government of Saskatchewan. Also, thanks to the Companion Animal Health Fund for the Graduate Tuition Scholarship Award and the Department of Veterinary Biomedical Sciences for the Graduate Student Scholarship Award.

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LIST OF ABBREVIATIONS

AA:	Amino Acid
AAFCO:	The Association of American Feed Control Officials
ALP:	Alkaline Phosphatase
ALT:	Alanine Aminotransferase
ATP:	Adenosine Triphosphate
AUC:	Area Under The Curve
BCS:	Body Condition Score
BW:	Body Weight
CDC:	Crop Development Center
CHF:	Congestive Heart Failure
CK:	Creatine Kinase
CO:	Cardiac Output
CP:	Crude Protein
CSA:	Cysteine Sulfinic Acid
DB:	Direct Bilirubin
DBP:	Diastolic Blood Pressure
DCM:	Dilated Cardiomyopathy
DM:	Dry Matter
DWT:	Left Ventricular Diastolic Wall Thickness
EDV:	Left Ventricular End-Diastolic Volume
EF:	Ejection Fraction
ESV:	Left Ventricular End-Systolic Volume
FB:	Fava Bean
FDA:	Food And Drugs Administration
FM:	Fermentation/Fermented
FMD:	Flow Mediated Dilation
G6PD:	Glucose-6-Phosphate Dehydrogenase
GE:	Gross Energy
GGT:	Gamma-Glutamyltransferase
GLDH:	Glutamate Dehydrogenase

HCO ₃ :	Bicarbonate
HMWDF:	High Molar Weight Dietary Fiber
HP:	High Protein
HR:	Heart Rate
HT:	High Tannin
IB:	Indirect Bilirubin
LDL:	Low Density Lipoprotein
LT:	Low Tannin
LVIDd:	Left Ventricular End Diastolic Diameter
LVIDs:	Left Ventricular End Systolic Diameter
MV:	Maximum Velocity
NP:	Normal Protein
NTproBNP:	N-Terminal Pro-Brain Natriuretic Peptide
RBC:	Red Blood Cell
ROS:	Reactive Oxygen
SAH:	S-Adenosylhomocysteine
SAM:	S-Adenosylmethionine
SBP:	Systolic Blood Pressure
SCD:	Sudden Cardiac Death
SV:	Stroke Volume
SWT:	Left Ventricular Systolic Wall Thickness
TB:	Total Bilirubin
UF:	Unfermented
USDA:	United States Department Of Agriculture
VBMS:	Veterinary Biomedical Sciences
VTI:	Velocity Time Integral
WBC:	White Blood Cell
WCVM:	Western College Of Veterinary Medicine
YGC:	Yeast Extract Glucose Chloramphenicol
YPD:	Yeast Peptone Dextrose

CHAPTER 1

INTRODUCTION

1.1 Overall Introduction

This thesis contains two research chapters written in manuscript-style formatting. Chapter three was published in *Frontiers in Veterinary Science* (Special Issue Research Topic: Nutrition and Management of Animals We Keep as Companions) “Reis, L. G., T. Morris, C. Quilliam, L. A. Rodrigues, M. E. Loewen, and L. P. Weber. 2021. The effect of fermentation of high- or low-tannin fava bean on glucose tolerance, body weight, cardiovascular function, and blood parameters in dogs after 7 days of feeding: comparison with commercial diets with normal vs. high protein. *Front. Vet. Sci.* 8. doi:10.3389/fvets.2021.653771”, and Chapter four will be submitted to the journal *Metabolites* (Special Issue: Effects of Diets on Protein and Metabolism in Companion Animals).

1.2 Rationale

Fava bean is a pulse considered a good source of protein, fiber, and starch. Besides having a well-balanced profile of essential amino acids, it is low in methionine and cysteine, which are considered limiting amino acids of pulse proteins, and taurine (Schuster-Gajzágó, 2004; Millar et al, 2019). Fava bean is not an approved ingredient for pet food and, therefore, there is scarce literature regarding the safety of its inclusion in dog food. The toxicity of fava bean is related to its anti-nutritional factors such as pyrimidine glucosides (vicine and convicine) (Liener, 1989) that can cause the development of favism (acute hemolytic anemia) in predisposed humans (i.e., with deficient activity of glucose-6-phosphate dehydrogenase enzyme) (Luzzatto and Arese, 2018a). However, such a condition has not been described in the available data in dogs. Fermentation has a role in the food industry as a flavour enhancer and food quality improvement bioprocessing technique (Kieliszek et al., 2017). Fermentation has also been reported to diminish vicine and convicine content as well as decreasing trypsin inhibitor and condensed tannins (Coda et al., 2015a). Grain-free pet foods are popular diets that often use legumes or pulses as a replacement for grains, but are under investigation by the US Food and

Drug Administration for a possible link to cases of canine dilated cardiomyopathy (DCM) (U.S Food and Drug Administration, 2019). However, a causal relationship has not been definitely demonstrated to date. Historically, taurine insufficiency has the most evidence supporting it as a cause of diet-related development of DCM (Fascetti et al., 2003a; Backus et al., 2006a). Taurine is important for cardiac function mainly due to its role in the reabsorption of calcium by the sarcoplasmic reticulum and in the sensitivity of the myofilaments to calcium (Bakker and Berg, 2002a). Moreover, taurine may allow cells to handle osmotic stress and is thought to inactivate free radicals (Huxtable, 1992). Taurine is not typically an essential amino acid for dogs as it can be endogenously synthesized from methionine and cysteine (Sanderson, 2006) although it can become conditionally essential. Therefore, the role of precursor amino acids needs to be considered along with taurine. Moreover, the threshold dietary levels that lead to development of DCM needs to be determined in both genetically predisposed dog breeds and in nutrition-related cases of DCM. Nutrients which can act as methyl donors and spare methionine requirements, additional supplementation of taurine to the diet, and the extent of cardiac oxidative damage might also play a role in that threshold and need to be acknowledged despite not being assessed in this thesis.

Grain-free, pulse-containing diets are expected to be high, specially in soluble fiber and oligosaccharides, which could increase fermentation of the sulfur-containing amino acids, leading to reduced bioavailability or higher fecal excretion in cats (Kim et al., 1996a; Kim et al., 1996b). Previous studies have reported that high fermentable dietary fiber in dogs increases the need for dietary taurine or taurine precursors (e.g., methionine and cysteine) due to increased fecal excretion of taurocholate (the predominant bile acid in dogs), leading to fecal loss of taurine (O'Máille et al., 1965; Story and Kritchevsky, 1978). Thus, this thesis will examine the short-term (Chapter 3; 7 days feeding) and longer-term (Chapter 4; 28 days feeding) effects of feeding diets formulated with 30% inclusion of two different varieties of fava beans (low- or high-tannin varieties), both with and without prior fermentation with *Candida utilis* on dog health. The short-term and long-term effects of feeding these diets will be compared to two specific commercial diets (a traditional moderate protein, grain-containing diet versus a newer higher protein, grain-free diet with more varied ingredients and taurine supplementation). End-points examined included: body weight, body condition score, glucose response, macronutrient

and amino acid (AA) digestibility of whole, extruded diets, cardiovascular health and overall health in adult beagle dogs.

1.3 Objectives

The overall objective of this research was to present an investigation of fava bean as a potential ingredient for dog foods and determine how its fermentation with *Candida utilis* could impact the nutritional value of the diet. Moreover, an evaluation of the comparison between commercial and the lab-formulated diets was designed to analyze how each diet would affect dog body weight, body condition score, glucose response, macronutrient and AA digestibility of diets, cardiovascular health and overall health. Finally, the two studies were contrasted to evaluate if the feeding time affected the results observed on each separated findings. Detailed objectives of each chapter are as follows:

Chapter 3:

Objective 3.1: Determine how short-term feeding (7 days) of fava bean-based diets (low or high tannin varieties) affect beagle glucose tolerance, body weight, cardiovascular function and blood parameters (red and white blood cells, chemistry panel and plasma amino acids levels).

Objective 3.2: Contrast two commercial diets (grain-containing and grain-free) to the same parameters described above.

Objective 3.3: Evaluate if fava bean is a safe ingredient to use in dog food for short-term feeding.

Objective 3.4: Analyze how fermentation of two varieties of fava bean with *Candida utilis* impacts dog health when used in dog food and fed for a short term.

Chapter 4:

Objective 4.1: Confirm/compare the changes that occurred after short-term feeding in the first study to a longer term (28 days) of feeding on glucose response, overall health and cardiovascular parameters, and plasma amino acids levels.

Objective 4.2: Measure fecal bile salts to investigate the possible effects of fiber levels on taurine utilization.

Objective 4.3: Analyze plasma levels of canine N-terminal Pro-Brain Natriuretic Peptide (NT-proBNP) and Cardiac Troponin I as biomarkers to screen for signs of myocardial dysfunction and possible occurrence of dilated cardiomyopathy (DCM).

1.4 Hypothesis

The overall hypothesis for this research was that pulse-based diets would impair cardiovascular health (showing DCM signs), potentially due to low taurine, cysteine or methionine levels and high soluble fiber content. Moreover, fermentation of fava bean flour with *Candida utilis* will improve diet quality and, consequently, dog health.

Chapter 3:

Hypothesis 3.1: Diets made with the high-tannin variety will have increased content of anti-nutritional factors, which will result in anemia and impaired glucose tolerance in dogs after 7 days of feeding each diet considering development of a favism-like disease (acute hemolytic anemia and glucose-6-phosphate-dehydrogenase deficiency).

Hypothesis 3.2: The fermentation of fava beans will reduce toxicity of fava bean in dogs

Hypothesis 3.3: Fermentation will improve glucose tolerance due to consumption by the yeast of rapidly available carbohydrates during the fermentation process.

Hypothesis 3.4: Fava bean-based diets with lower level of methionine and taurine, combined with higher fiber content will produce adverse cardiovascular changes.

Chapter 4:

Hypothesis 4.1: Cardiovascular indicators of DCM will be more evident after increased time of feeding each pulse-based diet (28 days), while longer term feeding of the normal protein commercial diet (a grain-containing diet) will support better cardiac health when compared to the high protein commercial diet. These findings will be coincident with decreased plasma levels of cysteine, methionine and/or taurine.

Hypothesis 4.2: High fiber diets would cause enhanced taurine utilization and corroborate with taurine insufficiency which, in turn, will be seen along with cardiac impairment.

Hypothesis 4.3: Elevations in NT-proBNP and cardiac troponin I plasma concentrations will provide evidence for adverse cardiac alterations.

Hypothesis 4.4: Unfermented fava bean-based diets will show more toxicity when compared to the fermented diets, primarily observed as impaired glucose tolerance and anemia.

CHAPTER 2

LITERATURE REVIEW

2.1 Fava Bean Production in Canada and the Pet Food Industry

Pulses, such as dry bean, pea, lentil, chickpea and fava bean, are defined by the Food and Agricultural Organization of the United Nations as “*Leguminosae* crops harvested exclusively for dry grain.” Fava bean (FB; *Vicia faba*), a protein-rich legume seed, also recognized as broad beans, field beans, bell beans, or tic beans, is native to North Africa and West Asia, but now with the largest production in China (Akibode and Maredia, 2012). The enthusiasm for fava bean in Western Canada started with establishing the European germplasm in the late 1960’s (Khazaei et al., 2021). Fava bean breeding was introduced, in the late 1970’s, by the University of Manitoba (UManitoba) and University of Saskatchewan (USask) but became inoperative in the 1990’s (Khazaei et al., 2021). In Canada as a whole, pulse production has been expanding since the 90’s as a result of the growing market opportunities, area enlargement, and the application of new technologies by the producers (Singh et al., 2013). From 1970 through 1971, fifteen different varieties were evaluated in Manitoba and Saskatchewan (Evans et al., 1972). As reviewed by Khazaei et al., 2021, the expansion occurred with more extensions introduced and tested from 1971 to 1973. In addition, more than 20 locations in western Canada and four European varieties were approved at that time. As the result of shorter growing seasons in the Canadian prairies, the spring-type varieties originating from the continental European zone, such as Germany and Austria, were the best-adapted germplasm at first. At the beginning of the 2000’s, some advanced cultivars (such as Snowdrop and Rodeo) were introduced and adapted to the Canadian prairie climate.

In 2002, the Crop Development Center (CDC) increased the fava bean program to combine specific attention on developing a germplasm base integrating small seed size and reduced anti-nutritional factor concentrations. Fava beans with small seeds, which are round-shaped, decrease the risk and seeding costs without waiving yield capability. Moreover, at that time, 1500 fava bean extensions were appraised in two different sites in Saskatoon. In 2008,

CDC started a breeding program with fava bean for the possible food and feed markets (Espinosa, 2018). Considering the world pulse production from 2011 to 2013, 60% of that total production was attributed to seven different countries. India led with 24.3% and was followed by Canada as the third major producer with 7% of the total output (Joshi and Rao, 2017). Nowadays, after wheat, barley, canola, and corn, pulses are recognized as the fifth largest crop in the country (Government of Saskatchewan, 2016). Since 1972, fava beans have been grown as a replacement for soybean meal for livestock diets in the prairie provinces of Canada. Saskatchewan grew nearly 40,000 hectare of fava bean from 2014 to 2017 (Penner, 2017). The principal genotypes grown in Saskatchewan in 2015 were the low tannin variety (small seeds) CDC Snowdrop and Snowbird as well as the normal tannin variety FB9-4, Taboar, Florent and SSNS-1 (Fleury and Baker, 2015).

According to the Global Dog and Cat's Pathfinder (Government of Canada, 2018), in 2017, the global pet care industry is worth USD 116.6 billion and the majority of this amount (USD 84.5 billion) was aimed at pet food consumption. In addition, Canada exported USD 525.3 million in dog and cat food, making Canada the eighth-largest exporter in 2017. Dog and cat food comprises 95% of the pet food industry, which was projected to grow up to 6% per year every year until 2022, with an estimated global pet food market growth value of USD 91 billion (Olatunde and Atungulu, 2018). In 2000, the United States Department of Agriculture (USDA) Federal Agricultural Services (FAS) showed that trends in western Canada made it an important destination for US pet food. The human population in the region was increasing and, consequently, the number of pets followed that occurrence. At the same time, Western Canadians were starting to be more concerned with health and nutrition, leading to higher interest in premium pet food and pet foods that include certain ingredients touted for better nutrition (Mcbride, 2003). Moreover, sales in the pet food market are mainly propelled by premium foods, often with natural, organic, and health claims. Novel ingredients such as peas, chickpeas, potatoes, and tapioca have been included in many new pet diets for years. Although a rare ingredient and not yet approved to be used in pet foods, fava beans have recently begun to be investigated (Corsato Avarenga et al., 2020).

2.1.1 Fava Bean Characteristics, Nutritional Value, and Anti-nutritional Factors

High nutritional value, simple preservation and low cost of production support the importance of legumes as an essential food. Fava bean is a winter-grown legume included in the

family Leguminosae. Moreover, it is mainly consumed in Egypt and India in the human food market, where pulses are used as a significant source of essential nutrients, particularly protein. In contrast, in developed countries, animal ingredients are the main source of protein (Rochfort and Panozzo, 2007; Ali et al., 2016).

Pulse crops are an important source of vitamins (folate, riboflavin and thiamine), minerals (iron and zinc), protein, and starch (Tiwari and Singh, 2012). The seeds are comprised of 20-25% protein (DM), 39-51% starch (DM) (high amylose content), and 1-2% oil (DM) (mainly polyunsaturated fatty acids) (Schuster-Gajzágó, 2004; Gopalan et al, 1999). Fava beans are rich in starch and protein. The cotyledons have a high weight and contribute to the whole amount of protein of a seed. Therefore, dehulling significantly enhances the protein levels of fava bean seeds (Alonso et al., 2000). Protein content can differ depending on the genotypes. In contrast, starch is the main macronutrient and energy source in pulses (approximately 42% DM). Generally, there is a negative correlation between starch and protein content in pulse seeds. Compared to cereal grains, fava beans have higher lysine levels, , diminished cysteine, tryptophan, and methionine, and lower fat and sugar levels (1% DM and 4% DM, respectively) (Crépon et al., 2010), but are also a good source of dietary fiber with a low glycemic index in people despite a high amount of carbohydrate (Xu et al., 2007). Moreover, legumes are also rich in water-soluble B vitamins (Hoppner and Lampi, 1993). Low tannin genotypes can have higher crude protein and higher sugar levels (Espinosa, 2018). However, the low tannin varieties tend also to have lower starch, fibre and fat content when compared to normal tannin genotypes. In humans, as described by Campos-Vega et al. (2010), fava beans offer a large number of benefits to their health due to phenolic compounds, oligosaccharides, enzyme inhibitors, phytosterols and saponins. Oligosaccharides were found at high levels when comparing dehulled fava bean to rice. Moreover, as the fava bean inclusion increased in different diets, so too did the oligosaccharides (Corsato Alvarenga and Aldrich, 2019).

While fava beans are approved as ingredients for feeding livestock, the Association of American Feed Control Officials (AAFCO) has yet to approve their use in pet foods over concerns about potential toxicity from anti-nutritional factors. Fava beans have numerous anti-nutritional factors such as condensed tannins, trypsin inhibitor activity, lectins and pyrimidine glucosides (vicine and convicine) (Liener, 1989).

Condensed tannins can precipitate protein and form soluble or insoluble complexes with many macromolecules (Frutos et al., 2004; Acuña et al., 2008; Addisu, 2016), and they are found in the seed coat of the fava bean. A high concentration of condensed tannins (>80 g/kg DM) can affect diet palatability (Rodriguez Espinosa, 2018). Considering effects of tannin content and fava bean nutritional value for pigs, Crepon et al. (2010) reviewed the ability of tannins to reduce energy and protein nutritional value but found it difficult to quantify the mechanism of effect of tannin. Variability may also happen because of interactions with other seed compounds, such as insoluble cell walls, which may vary with the type of the tannin.

Vicine and convicine have been regarded as anti-nutritional factors associated with favism occurrence in humans. However, there is no data available describing such disease in dogs fed fava bean-based diets. Favism is triggered in humans by the presence of the β -glucosides vicine and convicine and only develops in subjects with glucose-6-phosphate dehydrogenase (G6PD) deficiency (Luzzatto and Arese, 2018a). Such a condition produces acute hemolytic anemia, with hemoglobinuria and moderate to severe signs of anemia in humans with this G6PD deficiency if they consume fava beans with high levels of vicine/convicine (Luzzatto, 1973). Additionally, G6PD is responsible for the first step in the pentose phosphate pathway, converting glucose to ribose-5-phosphate, a major glucose metabolism pathway. So, in subjects deficient in G6PD it is possible that glucose is not fully utilized and may cause an impaired glucose tolerance. Chanmugam and Frumin, (1964) have reported an abnormal response to the oral glucose tolerance test in human adults deficient in G6PD. Interestingly, Arese and De Flora (1990) stated that β -glucosides are susceptible to inactivation when cooked, so the process of extrusion to produce dry kibble dog food could remove a large portion, but not all of these anti-nutritional factors. Hence, an impaired glucose tolerance as well as anemia, if found in the dogs of this present study, would be evidences that vicine and convicine are toxic elements present in fava bean and would preclude the use of fava beans in dog food.

2.2 Grain-Free Diets and Canine Dilated Cardiomyopathy

Grain-free dog foods are diets with no grain-based ingredients such as wheat, barley, corn, or rice. Grain-free diets are potentially the most well-known and best publicized pet-diet trend in which companies claim that these high-protein “ancestral diets” are healthier than the grain-containing pet foods. In the continual search for novel ingredients to replace grains,, pulses are

the most common replacement for grain in dog foods. Pulses are a group of legumes with a low concentration of lipids. While peas are the most commonly used pulse in commercial dog foods, more recently, fava bean has been explored as an alternative (Corsato Alvarenga et al., 2020).

The Food and Drug Administration (FDA) of the USA reported in July 2018 some cases of dilated cardiomyopathy (DCM) in dogs that were fed foods formulated with potatoes and pulse ingredients (i.e. grain-free diets). However, this FDA warning was based initially on only 9 case reports with no controls and, even now, the accuracy of this potential link has not been well established. No studies to date have definitively demonstrated that diet is the cause and, if so, which factors might be responsible. FDA officials announced in November 2020 that the agency was following the plan to work with researchers outside the organization on studies of nonhereditary DCM. The FDA also acknowledged that DCM was a complex multi-factorial disease affected by genetics, underlying medical conditions, and diet (FDA, 2020).

Taurine deficiency is known to affect DCM in many dog breeds (Kaplan et al., 2018) even though it is not considered an essential amino acid in dogs since it is endogenously synthesized. If there is a link between the inclusion of pulses in grain-free diets and taurine-deficiency-mediated DCM, it may be related to the dietary fiber provided by pulse-containing diets. These diets are hypothesized to be relatively high in total dietary fiber (TDF) and possibly providing different types of fiber compared to traditional pet food diets. Several previous studies have reported that increasing fermentable dietary fibre intake in dogs increases the need for dietary taurine or methionine/cysteine (taurine precursors) due to increased fecal excretion of taurocholate (the predominant bile acid in dogs) and the related fecal loss of taurine (O'Máille et al., 1965; Story and Kritchevsky, 1978). Having said this, direct evidence linking fiber from pulses to changes in cysteine, methionine, or taurine blood concentrations or to DCM is lacking in dogs.

2.2.1 Cardiomyopathy Incidence, Clinical Signs, and Possible Causes

Among cardiovascular diseases in dogs, up to 75% are related to chronic degenerative valve disease (Kahn, 2005). The most common primary myocardial disorder and the second most common heart disease in dogs is DCM (McCauley et al., 2020). Considering the total number of dogs in the United States as 77,000,000 (from a review by AVMA, 2018), the incidence studies (Fioretti and Delli, 1988) indicate that a minimum of 308,000 to 1,001,000 dogs in the US have DCM at any given period.

Historically, DCM is thought to be an inherited genetic condition. Currently recognized etiologies of DCM in dogs include genetic factors, tachycardia, taurine deficiency, toxic factors, and possibly carnitine deficiency (Sisson et al., 2000; Kittleson, 1998; Tidholm, 2001). Studies in North America have shown a higher incidence in large/giant breeds such as Doberman Pinschers, Irish Wolfhounds, Great Danes, Boxers, (Dukes-McEwan et al., 2003), Golden Retrievers and Saint Bernards (Fascetti et al., 2003a; Bélanger et al., 2005; Backus et al., 2006a; Vollmar et al., 2013). However, Golden Retrievers and American Cocker Spaniels also seem to have a predisposition to taurine deficiency (Kramer et al., 1995; Bélanger et al., 2005). DCM initiates with a subclinical manifestation phase because of a compensatory mechanism of the cardiovascular system, initially described in Doberman Pinschers (Calvert et al., 1997). In this occult phase, decreased systolic function and arrhythmias may occur, although no clinical signs may be present. Thus, early screening in predisposed breeds is important (Vollmar, 1999). Also, in the occult phase, other abnormalities that may be present are increased left ventricular and atrial dimensions, decreased myocardial contractility, and ventricular premature complexes. The duration of this phase is variable and known to last for months to years. Throughout this asymptomatic phase, progressive heart enlargement and arrhythmias occur (Oyama, 2008). With the natural development of the disease, the heart decreases its ability for contractile function, generating a decreased ejection fraction and cardiac output. Moreover, uninterrupted myocardial remodelling may lead to dangerous arrhythmias. The signs of the disease occur when exercise intolerance, congestive heart failure (CHF), syncopal episodes, and sudden cardiac death (SCD) are observed (O'Grady and Horne; 1992). Frequently, SCD occurs as the first sign of DCM and can happen in up to 40% of Doberman Pinschers (Koch et al., 1996). Besides SCD, an advanced congestive heart failure that is refractory to medical therapy is also a cause of death. Arrhythmias in the form of premature ventricular beats, ventricular tachycardia, and atrial fibrillation are common in this clinical phase (Oyama, 2008). Ideally, all dogs should receive an electrocardiogram (ECG) to detect arrhythmias or evidence of heart enlargement; however, a normal ECG does not rule out DCM. Chest radiographs are relatively insensitive to mild increases in the heart size but may be useful when monitoring during the later progression of the disease. In contrast, echocardiogram (cardiac ultrasound) is most useful to quantify heart enlargement and systolic function since it can detect pre-clinical DCM as well as monitor progression to the clinical phase. Finally, urinalysis, and serum chemistry aid in clinical

monitoring to identify azotemia, electrolyte abnormalities, plasma levels of natriuretic peptides (NT-proBNP), and cardiac troponin-I forms (Oyama, 2008). NT-proBNP is released in response to atrial stretch and elevations in the blood are correlated to severity of heart failure in dogs and thought to initiate remodelling changes in the heart during DCM (Oyama et al., 2008b; Wess et al., 2011). In contrast, cardiac troponins are contractile proteins released into the bloodstream when cardiomyocytes die and thus are markers of acute cardiac damage (Ljungvall et al., 2010).

When genetic predisposition is not fully responsible for dogs developing DCM, then diet, physiology, and/or exposure to toxic factors are other parameters that may instead trigger the disease (Mansilla et al., 2019) which was acknowledge by FDA in 2020 when was said that DCM is a complex multi-factorial disease like previously cited in this review (FDA,2020).

2.3 *Candida utilis* fermentation

Yeasts are a collection of microorganisms that do not have especially high nutritional requirements, show high levels of growth, and their cultivation does not usually rely on geographic or environmental conditions (Santos et al., 2013). In general, yeast fermentation is a typical bioprocessing technique used as a food quality and flavour enhancer (Kieliszek et al., 2017). Moreover, the process can improve texture, appearance, shelf-life, and nutrient quality (Frias et al., 2017), while at the same time can decrease non-nutritional compounds found in legume seeds such as protease inhibitors, oligosaccharides, phytate, and lectins (Deshpande et al., 2000). The fermentation process promotes positive biochemical alterations in nutritional composition by the microorganisms participating, with the simplest fermentation approach utilizing the natural microbiota existing on the legume surface, while more advanced approaches initiate fermentation with starter cultures (Harlander, 1992). The genus of yeasts recognized as *Candida* was first separated in 1923 by Christine Nerckhout based on specific morphological, biochemical, and physiological characteristics (Barnett, 2004). *Candida utilis* is a type of yeast that grows in different substrates, is not inhibited by high concentrations of sugars, and does not produce alcohol in its process. The yeast has important nutritional value by itself, while also considered rich in proteins, nucleotides and vitamins. Glucanase was isolated from cell walls of *C. utilis* yeasts (Drábiková et al., 2009) which prevents the formation of reactive oxygen forms (ROS) in *in vitro* and *in vivo* systems (Krizková et al., 2001; Drábiková et al., 2009). Adding yeast extract or fermenting an ingredient with yeast can also produce a final product with

increased quality and superior flavour (Buerth et al., 2016; Kieliszek et al., 2016). Rajoka et al. (2012) showed that the concentration of lysine after cultivating *C. utilis* and *Brevibacterium lactofermentum* bacteria ranged up to 25%, while the protein level in the biomass was 32%.

C. utilis yeasts are usually utilized to produce many preparations, such as protein hydrolyzates or yeast extracts. The awareness to the value of the cellular protein of feedstock yeasts is a consequence of their well-balanced amino acid composition. A suitable amino acid composition is needed in order to guarantee adequate growth and development, especially in young animals (Kurcz et al., 2018). According to Zalacain et al. (1996), the lipolytic abilities of the *Candida* yeasts affect organoleptic properties and improve the shelf life of the products. Additionally, Coda et al. (2015) reported that fermentation with *Lactobacillus plantarum* used in fava bean flour caused a reduction of vicine and convicine levels by more than 91% and significantly decreased trypsin inhibitor activity and condensed tannins. The aglycones divicine and isouramil, which are breakdown products of vicine and convicine, were also degraded by *Lactobacillus plantarum* fermentation, confirming that the process has potential to produce a fava bean flour that is favism toxicity-free (Rizzello et al., 2016a).

2.3.1 Nutritional Value

The microorganisms included in pulse fermentation hydrolyze and metabolize seed components leading to value-added products. These microorganisms also have the capacity to add antimicrobial compounds to a feed ingredient after fermentation as well as advantageous organic acids that can maintain the food or feed by inhibiting the growth and survival of harmful microflora (Frias et al., 2017). The active yeast fermentation of several types of cowpeas, peas, and kidney beans increased the protein chemical score and essential amino acid index, resulting in improved overall protein quality (Khattab et al., 2009). Coda et al. (2015) reported that a lactic-acid fermentation of fava bean resulted in increased levels of essential amino acids and augmented the *in vitro* protein digestibility. *C. utilis* fermentation of lupin seeds increased the crude protein level by 13.5 and 26% when compared to raw seeds of two varieties of lupin (Kasprowicz-Potocka et al., 2015). The same study showed that the content of lysine, cystine, and threonine in the fermented seed dry matter was higher than in the raw product. Besides that, Kasprowicz-Potocka et al. (2015) also reported that the total raffinose family oligosaccharides (RFOs), an anti-nutritional component that can reduce the utilization of nutrients from low-alkaloid lupin seeds (Zdunczyk et al., 1998), was completely removed from the seeds after the

fermentation with *C. utilis*. The bacterial transformation of carbohydrates, proteins and antinutritive components included in raw lupin seeds results in the formation of an increased number of compounds that may have beneficial or adverse effects on human and animal health. RFOs are not hydrolyzed by digestive enzymes of monogastric animals. Instead, bacterial α -galactosidase in the large intestine hydrolyze RFOs into gases, which can affect nutrient absorption (Zdunczyk et al., 1998) and result in flatulence and diarrhea (Seve et al., 1989; Sobotka et al., 2013). On the other hand, oligosaccharides and polysaccharides are known as potential substrates for fermentation by beneficial intestinal bacteria as well which can trigger their growth and action (Bartkiene et al., 2013).

Fermented fava bean flour has higher *in vitro* protein digestibility and lower trypsin inhibitor activity when compared to raw flour (Chandra-Hioe et al., 2016). Still, the protein digestibility could be caused by the soaking and dehulling processes that the pulse went through. Fermented pulses offer a significant source of carbohydrates (50-70% DM) (Abu-Salem and Abou-Arab, 2011). While Chandra-Hioe et al. (2016) did not present a significant difference for water absorption when comparing raw and fermented fava bean flours, Raikos et al. (2014) reported that parameter to be 1.5 g/g, which was higher than wheat flour. Moreover, the authors showed that fava bean flour had an improved capacity to bind more water than wheat flour, which could be related to its higher protein level. The water absorption capacity was measured to establish the gelatinization of the starch present in the flour. Microbial and endogenous pulse amylases participate importantly in pulse fermentation processes and were reported to increase their activities in the early stages but declined gradually with the fermentation development (Soni et al., 1985). In addition, the hydrolysis of starch, amylose and amylopectin, diminish progressively throughout the fermentation and result in reducing sugars (Soni and Sandhu, 1989; Audu and Aremu, 2011; Sotomayor et al., 1999). Many studies with lactic-acid fermentation reported that the process increases the starch digestibility (Vidal-Valverde et al., 1993; Yadav and Khetarpaul, 1994; Bhandal, 2008) and similar results were found for yeast fermented pulses (Starzynska-Janiszewska et al., 2012; Abu-Salem and Abou-Arab, 2011). Regarding fiber fractions, a decrease in soluble dietary fiber was documented in fermented black beans, green grams (mung beans), cowpeas, and Bengal grams (type of chickpea); whereas, insoluble dietary fiber increased, resulting in an overall increase of total dietary fiber after fermentation (Veena et al., 1995; Granito and Álvarez, 2006).

2.3.2 Health Benefits and Concerns

Positive effects of fava bean consumption by humans include reported decreases in plasma LDL-cholesterol levels (Frühbeck et al., 1997), high protein and fibre levels, and energy supply (Coda et al., 2015a). In general, plant-food-based diets are regarded as part of a healthy diet, slowing or preventing progression of chronic diseases such as obesity, diabetes, and cardiovascular diseases in humans (Pistollato and Battino, 2014). Moreover, some notable health benefits attributed to phytochemicals from pulses are linked to potential antioxidant action and anticancer activity (Rochfort and Panozzo, 2007). Carciofi et al. (2008) also showed that diets containing pea and lentils are advantageous compared to diets containing corn, brewer's rice and cassava flour in regards to glucose tolerance in dogs. Canine health problems related to glucose intolerance, such as obesity and diabetes mellitus, resulted in diet investigations regarding glycemic control. Thus, diets that postpone and lengthen glycemic and insulinemic responses and consequently decrease plasma fluctuations are beneficial (Graham et al., 1994). Likewise, Adolphe et al. (2015) found that pea diets are a healthier dog food option compared to rice diets in terms of producing improved metabolic health, specifically reduced postprandial insulin responses, in obese dogs.

As mentioned, fava beans can cause favism, observed as acute hemolytic anemia with hemoglobinuria and moderate to severe signs of anemia, in predisposed humans due to the β -glucosides vicine and convicine (Luzzatto and Arese, 2018a). However, a similar disorder is not proven to occur in dogs. Therefore, further investigations have been pursued. Corsato Alvarenga et al. (2020) fed dogs with diets containing 0, 10, 20, and 30% inclusion of fava bean for 14 days. The animals observed had no health issues and no impact on blood cell count or serum biochemistry panel, including no signs of anemia or abnormal hematology. Vicine and convicine, after hydrolysis transformation, generate the aglycones divicine and isouramil, respectively (Crépon et al., 2010). Although vicine and convicine can be deactivated after processes such as cooking, seed drying, and extrusion, divicine and isouramil can yet be created through microbial β -glucosidases throughout digestion in the large intestine and cecum in humans (Yannai and Marquardt, 1985; Corsato Alvarenga and Aldrich, 2019). Therefore, favism can be stimulated by consuming both raw and cooked fava beans if the pyrimidine glycosides are not completed hydrolyzed prior to ingestion (Rizzello et al., 2016a). When fermenting with *Lactobacillus plantarum* as a starter fava bean flour, vicine and convicine were decreased by

more than 90%, and the content of other anti-nutritional factors was positively impacted (Coda et al., 2015a). Rizzello et al. (2016) showed that after spontaneous fermentation or fermentation generated by *L. plantarum*, the aglycones of vicine and convicine were rapidly degraded, which is encouraging for humans suffering from favism, since fermented fava bean flour was proven safe for consumption. Also, after a hemolytic assay using normal red blood cells, the hemolysis resulting from the extract of lactic acid bacteria fermented fava bean was less than half the spontaneously fermented control. This outcome suggests the detoxification of the fava bean after fermentation.

2.4 Taurine, Its Precursors, and Diets in Dogs

Taurine, 2-aminoethanesulfonic acid, is a sulfur-containing beta amino acid which occurs in a wide variety of mammals (Jacobsen and Smith, 1968) in free form or conjugated with bile acids. Distinct from most of other alpha amino acids, taurine is not incorporated into protein but is one of the most abundant free amino acids in the body (Sanderson, 2006). Taurine is synthesized endogenously from cysteine in the liver of dogs. Moreover, taurine is present in high concentrations in cardiac and skeletal muscle, the central nervous system, and platelets (Tenaglia and Cody, 1988). Taurine is an essential amino acid in cats but not in dogs (Sanderson, 2006).

Methionine is a sulfur amino acid essential for protein biosynthesis that can also be transsulfurated to form cysteine (Shoveller et al., 2005). Cysteine is a nonessential sulfur-containing, methionine-dependent amino acid in mammals. Cysteine can be catalyzed by cysteine dioxygenase to form cysteine sulfinic acid (CSA) which in turn results in taurine by CSA decarboxylation (Ferzola, 2019). Most mammals require taurine and glycine (with the ideal glycine:taurine ratio as 3:1) to conjugate primary and secondary bile salt. In addition, most mammals can utilize glycine to conjugate bile salts in cases when taurine is low in serum, in case of hepatobiliary and ileal dysfunctions (Garbutt et al., 1969). However, cats (Hickman et al., 1990) and dogs (E. R. O'Máille et al., 1965) are unable to conjugate bile salts with glycine; thus, these two species conjugate bile salts with only taurine. Before the 1970's, taurine was hypothesized to be a biochemically inert end product of methionine and cysteine metabolism, as well as inorganic sulfate. Since then, taurine is recognized as indispensable for animal tissues despite the fact that it is not incorporated into proteins or used for protein synthesis.

Methionine has three major metabolic functions: transmethylation, to act as a primary methyl donor; transsulfuration, to form cysteine; and protein synthesis (Shoveller et al., 2005)

(Figure 2.1). Dietary methionine requirements can be reduced, potentially by 50-80%, when there is sufficient dietary cysteine (Shoveller et al, 2005). The first step of cysteine synthesis is the transmethylation of methionine to form homocysteine. This process forms a primary methyl donor, S-adenosylmethionine (SAM; aka S_{AM}e), that methylates compounds to create products like creatine and phosphatidylcholine, leading in the product of methylation homocysteine being formed; SAM might also be decarboxylated producing decarboxylated SAM, which then donates an aminopropyl group for polyamine formation after which methionine will be reproduced. The polyamines, with a methyl donor being released and converted in S-adenosylhomocysteine (SAH) (Shoveller et al., 2005) is hydrolyzed to form adenosine or homocysteine, which is a non-protein that differs from cysteine by the additional methylene group (Ferzola, 2019). Homocysteine can be remethylated to methionine or proceed to the transsulfuration pathway reacting with serine to form cystathionine (Shoveller et al., 2005). Cystathionine, in turn, can form cysteine or be directed to another route and form glutathione (Ferzola, 2019). Cysteine has its concentration in the body regulated by cysteine dioxygenase (CD), which has high activity in the liver and adipose tissues. When cysteine is available in the body, CD catalyzes a reaction to convert cysteine to cysteine sulfinic acid (CSA), which can be utilized in two pathways: a) CSA decarboxylation to form taurine or b) CSA transamination to form pyruvate and sulfite. In the first reaction, which occurs in the liver and depends on the substrate concentration, CSA is oxidized to cysteic acid, and can be decarboxylated to hypotaurine, which is oxidized to produce taurine. Moreover, cysteic acid can be transaminated to B-sulfinyl pyruvate. In the second reaction, CSA is transaminated to form glutamate (3-sulfinylpyruvate reacting with α -ketoglutarate), which will form pyruvate and sulfite (later oxidized to sulfate by sulfinic oxidase) (Ferzola, 2019). Due to a high correlation between hepatic CD levels and cysteine availability, changes in methionine intake may affect its stabilization. For example, dogs and cats produce proteasome inhibitor 1 to stabilize CD when fed low protein diets (Ferzola, 2019). The proteasome 1 degrades CD and produces CD intermediates to supply to the liver. Stipanuk et al. (2009) showed that the proteasome inhibitor 1 can increase hypotaurine accumulation and reduce taurine formation.

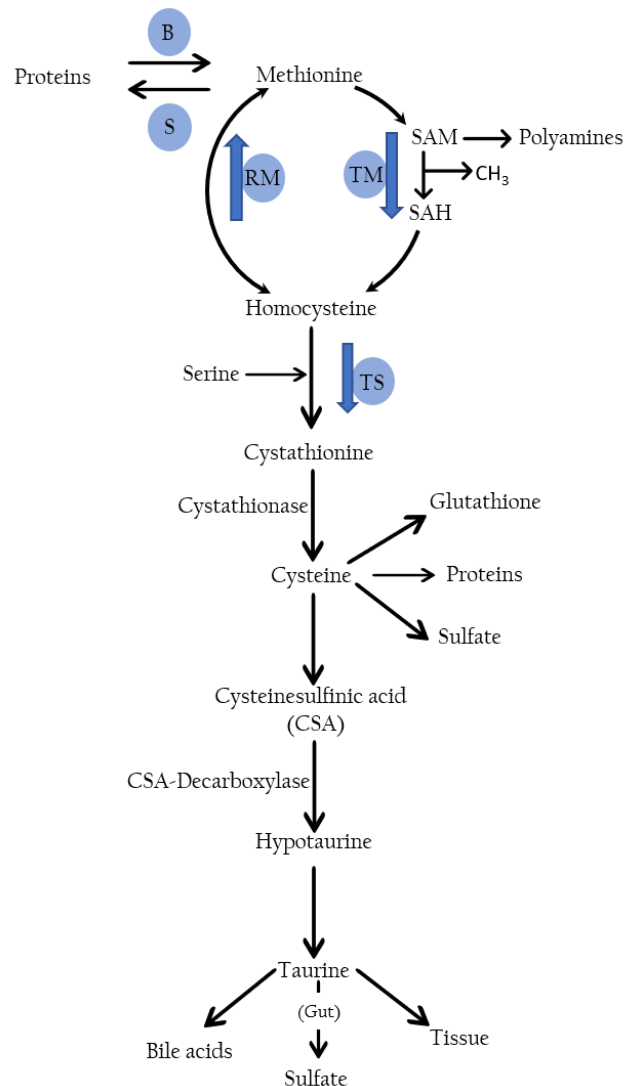


Figure 2.1 Schematic representation of transsulfuration pathway from methionine for the biosynthesis of taurine (Adapted from Hayes and Trautwein (1989) and Shoveller et al. (2005)). TM: transmethylation; TS: transsulfuration; RM: remethylation; S: protein synthesis; B: breakdown.

Diets with low concentrations of both taurine and sulfur-containing amino acid precursors have been linked to taurine-deficient DCM. Certain diets made for management of urate stones were reported to be associated with DCM, likely due to the low concentration of essential and nonessential amino acids, including crucial precursors for carnitine and taurine formation, in these low protein diets (McCauley et al., 2020). L-carnitine is a molecule soluble in water obtained by dogs through their diets or endogenous synthesis from lysine and methionine in the liver (Sanderson, 2006). In addition, iron, vitamin C, and vitamin B6 are required as

cofactors for the synthesis of carnitine (Bremer, 1983). Levocarnitine, which is a natural and biologically active amino acid derivative, plays a relevant role in lipid metabolism and mitochondrial defense. L-carnitine stimulates mitochondrial β -oxidation of long-chain fatty acids by promoting their passage through the inner mitochondrial membrane (Bremer, 1968). The mitochondrial long-chain fatty acid shuttle that facilitates beta-oxidation depends on both carnitine and the combined activity of three carnitine-specific enzymes. These enzymes require carnitine shuttling due to the cellular membrane impermeability of long-chain acyl-CoA.

Low-protein diets with cysteine and methionine concentrations at or above minimum requirement established by AAFCO, were also studied in 2001 by Sanderson et al. In this study, the dietary treatments were assigned for each dog for 48 months and caused significant decreases in whole blood taurine levels when compared to baseline levels. Supplementation with taurine and L-carnitine resulted in reversion of DCM clinical signs and increased life span of dogs. One dog with taurine deficiency progressed to DCM. This was the first study in dogs to clearly show that taurine deficiency preceded DCM (Sanderson, 2006). In another study, two unrelated dogs developed taurine deficiency when fed a low-protein tofu-based diet, even though the AAFCO requirements for protein were met. DCM may be caused by the lack of taurine and low concentration in sulfur-containing amino acids found in soybean-cure, the raw material of tofu (Spitze et al., 2003a). If this were true, then diets high in pulse content would potentially pose similar problems and is one way that taurine could be one mechanism linking grain-free diets and DCM in dogs.

2.4.1 Role of Taurine on Bile Salts Formation and Its Excretion

Bile, which has many components including bile acids, bilirubin, phospholipids, and cholesterol, is synthesized in the liver. Bile acids, responsible for 70% of the reactions related to bile synthesis, can react with specific amino acids such as glycine and taurine to form bile salts in most species (Ferzola, 2019). Bile salt synthesis can be divided into three processes: hydroxylation, conjugation of bile acids with taurine or glycine, and secondary bile acids formation. In hydroxylation, the cholesterol reaches the liver where it can be involved in two reactions: formation of chenodeoxycholic acid or cholic acid. Subsequently, in conjugation, the primary bile acids can be conjugated with taurine or glycine to form bile salts. (Ferzola, 2019). The conjugation reactions in dogs and cats, however, occur almost exclusively with taurine (Hickman et al., 1990; Mansilla et al., 2019a). Then, taurine reacts with chenodeoxycholic acid

and cholic acid forming two bile salts: taurochenodeoxycholic and taurocholic acid. If the reaction with taurine does not occur, the primary bile acids go to the intestine and then will be absorbed or converted into secondary bile acids in the small intestine. In the small intestine, the primary bile acids can be dehydroxylated by bacterial enzymes. After being metabolized, they are converted to secondary bile acids that can be used to emulsify fat or return to the liver through the enterohepatic circulation. Once the dihydroxylation occurs, two new bile salts are synthesized: the deoxycholic acid and the lithocholic acid. These salts can be conjugated with taurine in dogs and cats (or glycine in other species) to form taurodeoxycholic acid and tauroolithocholic acid (Ferzola, 2019). Most of the secondary bile acids are reabsorbed in the jejunum and ileum and return to the liver via the portal vein to complete the enterohepatic circulation (Hickman et al., 1990; Ajouz et al., 2014). Any changes in this recycling route may result in fecal losses of bile acids and taurine (Pezzali et al., 2020a).

It has been suggested that fiber or luminal pH can impact bile acid excretion composition and solubility (Garcia-Diez et al., 1996; Stratton-Phelps et al., 2002). Herstad et al. (2018) also reported that dietary composition can impact fecal bile acid profile in dogs. Furthermore, alterations to the intestinal microbial community may influence bile acid composition as deconjugation of primary bile acids can occur by the action of the commensal microbial enzyme bile salt hydrolase. This deconjugation phase works as an opening reaction for the dihydroxylation of primary bile acids in the colon through an bacterial enzyme to form secondary bile acids (Long et al., 2017).

2.4.2 Fiber Effects on Taurine Excretion

Typical ingredients used to increase fiber level in pet food include cellulose, beet pulp, corn fiber, rice bran, whole grain, and pulse fibers (de Godoy et al., 2013). Overall, the beneficial effects of fermentable and soluble fibers on health are associated with increased digesta viscosity, delaying gastric emptying, increasing satiety, decreasing rate of glucose uptake, lowering blood cholesterol levels, and facilitating gut microbiota growth (German et al., 1996; Brennan and Cleary, 2005; Jenkins et al., 2008). A study reported that 7.5% of inclusion of beet pulp was a sufficient dietary fiber to enhance the diversity of the fecal microbiota, with no adverse effects on nutrient absorption (Middelbos et al., 2010). This is relevant since the gastrointestinal tract was identified, in pig models, as the main location where sulfur-containing amino acids are metabolized (Bauchart-Thevret et al., 2009). The sulfur-containing amino acids,

methionine and cysteine, when metabolized in the intestine can highly impact the level in the plasma (McCauley et al., 2020). Moreover, these amino acids have been reported to support epithelial cells and gut action (Bauchart-Thevret et al., 2009). Fiber is a crucial nutrient in the diet that can increase gut health status depending on the category of the fiber and the intake quantity. The balance of the necessary amount and type present in the diet is vital for the best dog nutrition (Mansilla et al., 2019a).

Fiber is a category of nutrient that covers a large variety of compounds, with different chemical compositions, structures, sizes, and behaviours, but all are similarly indigestible. Fiber and the gastrointestinal tract work in cooperation to affect motility, digestion, physiology, the immune system, and metabolic functions. Dietary fiber (DF) includes non-carbohydrate compounds such as lignin and other carbohydrates that escape digestion by intestinal enzymes such as non-digestible oligosaccharides (NDO) and resistant starch (RS) (Klurfeld et al., 2018). There are relevant divisions in the fiber category such as viscosity and fermentability, although molecular weight and solubility are more often assessed because these latter attributes are easier to analyze. In terms of molecular weight, DF can be classified as high molecular weight DF (HMWDF) which includes fiber with degree of polymerization (DP) ≥ 10 ; or low molecular weight DF (LMWDF) which encompasses soluble NDO (DP of 3-9) (McCleary and Cox, 2017), many of which are treated as prebiotic fibers. On the other hand, the solubility category refers to the capacity to dissipate in water. Insoluble DF (IDF), usually extracted from plant cell walls, are in general non-viscous and less fermentable fiber that adds bulk. This can dilute calories, increase food volume and increase fecal output. When coarsely ground, IDF stimulates colonic motility, it also stimulates secretion of water and secretion of mucous to soften feces. However, when finely ground, IDF may instead be constipating (Williamns et al., 2019). Soluble DF (SDF) can also contribute calories and add bulk. However, SDF behaviour in the gastrointestinal tract and effects on fecal output are more complex because of diversity in viscosity and fermentability of different fibers. RS may play a role as IDF in the upper gastrointestinal tract, but it is usually a moderate or highly fermentable fraction, so its action in the lower gastrointestinal tract will differ from most other IDF. The ability of fiber to form gel-like compounds determines the viscosity which can be changed by local gastrointestinal tract conditions such as pH and ions, the form ingested and meal composition. The viscosity changes flux and mechanical mixing of digesta, altering the contact with the gastrointestinal tract, which modifies nutrient dispersion, digestion,

and absorption (Jha et al., 2019; NRC, 2006). For example, high viscosity delays gastric emptying and transit time in the small intestine (Fahey, 2005). Regarding fermentability, some fibers can be fermented by gut microbiota in the lower intestine, which may alter the composition of gut microbiota or metabolite formation (Wernimont et al., 2020). The fibers utilized by intestinal microflora that result in health benefits are classified as pre-biotic. Fermentation of fiber may form lactate, gases, and the short-chain fatty acids (SCFA) such as acetate, propionate, and butyrate. Generally, SCFAs are crucial in the balance of health and response to disease. On the other hand, at high concentrations, they can be disruptive, and over-accumulation may play an osmotic effect (NRD, 2006; Liu et al., 2021).

Nevertheless, high fiber concentrations can diminish apparent crude protein digestibility (Wilfart et al., 2007). Also, some dietary fibers such as non-starch polysaccharides have been reported, in pigs, to be relatively nonfermentable and instead have anti-nutritive outcomes (Cadogan and Choct, 2015). This can result in a decrease in sulfur-containing amino acid absorption and consequently in deficiencies of nutrients such as taurine. The possible decrease in protein digestibility caused by high dietary fiber can indirectly impair digestibility of taurine, carnitine, and other nutrients (McCauley et al., 2020). As previously discussed, such nutrients are crucial for regular function of cardiac muscle. This was reported in medium and large breed dogs fed beet pulp (Ko and Fascetti, 2016). The rationale was that the diets, even though formulated to meet AAFCO requirements, did not meet dog nutritional requirements because of the negative effects of fibers on nutrient absorption. In addition, fiber can impact fermentation by-products from microbiota in the hindgut and inhibit reabsorption of taurine, despite taurine being biosynthesized in sufficient quantities (Ko and Fascetti, 2016). Furthermore, higher levels of dietary fiber expand the size of the gastrointestinal tract in pigs and poultry (Nyachoti et al., 2000), increasing nutrient usage in this organ (Mansilla et al., 2019). On average, the gastrointestinal tract utilizes 30% of the dietary indispensable amino acid during absorption, and this utilization represents ~50% for sulfur-containing amino acids (Stoll et al., 1998; Mansilla et al., 2018), as concluded in pigs. Consequently, this increased demand in the gut reduces precursor availability for taurine biosynthesis and enhances the risk for taurine deficiency.

It was hypothesized by O'Máille et al. in 1965 that high-fiber diets can increase the chances of taurine deficiency by two mechanisms of action associated with obligatory bile acid conjugation with taurine in dogs and dependence on enterohepatic circulation for the

reabsorption of taurine and bile acids. Also, Garcia-Diez et al. (1996) and Stratton-Phelps et al. (2002) suggested that some fiber may bind bile acids in the intestinal lumen causing higher excretion rates of these metabolites; thereby, diets with high concentration of fiber may increase fecal output and depletion of taurine-conjugated bile. This process would demand a higher production rate of bile in the liver and, therefore, larger utilization of taurine (J. A. Story and Kritchevsky, 1978). Also, fermentable fibers may impact the population of the microbiota that degrade taurine in the intestinal lumen (Kim et al., 1996a; Kim et al., 1996b). In addition, luminal pH can be modified by the end-products of fermentation, including short-chain fatty acids, which can then affect bile acid excretion and solubility (Pezzali et al., 2020a). Increased excretion, either combined or not with degradation of taurine, from high fiber-containing diets may diminish enterohepatic circulation and recycling of taurine. Some fiber may affect gastrointestinal losses by the fermentation process. Dogs are deficient in some enzymes required to digest legume oligosaccharides that are transported to the lower intestine and are fermented by the colonic bacteria (Jezierny et al., 2010). Considering taurine as the sole amino acid utilized for bile acid conjugation in dogs, in the long-term, high-fiber diets could aggravate the risk of taurine insufficiency (Mansilla et al., 2019a). Thus, when feeding high-fiber diets, the supplementation of specific nutrients, such as taurine and other sulfur-containing amino acids, might be positive to avoid nutrient deficiencies (Mansilla et al., 2019a).

In summary, this thesis aims to investigate the effects on health when dogs are fed fava bean-based diets or commercial diets with different levels of protein inclusion after a 7-day feeding period versus 28-day feeding. Moreover, this thesis aims to examine how the fermentation with *C. utilis* of fava bean flours could improve the nutrition and health benefits in beagles. Finally, the potential relationship between taurine, sulfur-containing amino acids, bile acids and cardiac health in dogs consuming these diets will be examined in this thesis.

CHAPTER 3

THE EFFECT OF FERMENTATION OF HIGH- OR LOW-TANNIN FAVA BEAN ON GLUCOSE TOLERANCE, BODY WEIGHT, CARDIOVASCULAR FUNCTION, AND BLOOD PARAMETERS IN DOGS AFTER 7 DAYS OF FEEDING: COMPARISON WITH COMMERCIAL DIETS WITH NORMAL VS. HIGH PROTEIN

3.1 Preface

Chapter 3 is the short-term study contained in this thesis on fava bean and fermentation as pet food ingredients and its effects on dog health. Similar to the longer-term study found in the next chapter, eight beagle dogs were examined. This current chapter investigates the effects of fermentation of two varieties of fava bean on overall and cardiovascular health in beagles after seven days of feeding combined with a comparison between two commercial pet foods.

This chapter has been published in the *Frontiers in Veterinary Science*, with the following authors listed: Luciana G. Reis (responsible for the study design, the fermentation process, the animal work and tests, the biochemical examinations excluding those sent to specific labs for analyses, the data analyses, and the writing), Tressa Morris (responsible for the fermentation process, and helped with the animal work), Chloe Quilliam (helped with animal work), Lucas A. Rodrigues (responsible for the data statistics), Mathew E. Loewen (responsible for the study design and supplied with the fava bean flours), and Lynn P. Weber (supervised the studies, responsible for the study design, and for the writing).

3.2 Abstract

Fava bean, which is available in high- and low-tannin varieties, is not an approved pet food ingredient and was not included in the “assumed to be safe” category based on its ability to cause favism and hemolytic anemia in susceptible humans. The effects of 7-day feeding of test canine diets containing moderate protein (~27% DM) were compared with two control commercial diets with normal (NP, grain-containing, ~25% protein DM) or high protein (HP, grain-free, ~41% protein DM). Fava bean diets were formulated either with or without *Candida utilis* fermentation

processing to reduce anti-nutritional factors. Glucose tolerance, body weight, cardiovascular function, and blood parameters were investigated in beagles fed the NP or HP diets or a randomized, crossover, 2×2 Latin square design of the fava bean diets: unfermented high-tannin (UF-HT), fermented high-tannin (FM-HT), unfermented low-tannin (UF-LT), and fermented low-tannin (FM-LT). After 7 days, HP decreased red blood cells (RBC) ($P < 0.05$) compared with NP, while FM increased RBC compared with UF. HP increased blood bicarbonate, calcium, phosphorus, urea, cholesterol, and albumin:globulin ratio while decreasing bilirubin, liver enzymes, and total protein. Sodium:potassium ratio was increased in UF-HT, decreased in FM-HT, and intermediate in LT regardless of fermentation. Blood phosphorus was increased in HT. Blood amylase was increased in FM-HT and decreased in FM-LT, being intermediate in UF regardless of fava bean variety. Blood direct bilirubin was decreased in HT regardless of fermentation. Of note, left ventricular end-systolic volume and cardiac output were increased in NP compared with HP-fed dogs, but were normal and had no significant differences among the fava bean diets. As expected, plasma taurine, cystine, and cysteine levels were increased in HP-compared with NP-fed dogs. Plasma cysteine levels were increased in HT-compared with LT-fed dogs and in FM-compared with UF-fed dogs. Taken together, these results show that fava bean appears to be safe as a dog food ingredient at least in the short term, and its nutritional value appears improved by fermentation. Moreover, blood chemistry parameters and cardiovascular function were impacted by protein content which merits further investigation with longer term feeding trials.

3.3 Introduction

Fava bean (*Vicia faba* L.) has been regarded as a healthy, sustainable alternative for partially replacing animal protein sources in human diets (de Boer and Aiking, 2011). The varieties of fava bean are divided by their tannin levels such as low or normal/high tannin content which affects taste (Fleury et al.). Because pet owners are increasingly matching their own nutritional choices with that of their pets, incorporation of fava beans in pet diets as a source of carbohydrates and protein has been considered. Fava bean safety in dogs has been scarcely explored and is not approved as a feed ingredient for pet food by the Association of American Feed Control Officials (AAFCO) yet due to concerns about potential toxicity from anti-nutritional factors (Rahate et al., 2021a). Further complicating the approval of a novel pulse ingredient such as fava beans for use in pet food, the US Food and Drug Administration (FDA)

reported in July 2018, cases in which dilated cardiomyopathy (DCM) was observed in dogs fed grain-free diets, i.e., food formulated with potatoes and pulse ingredients instead of grains (Medicine, 2020). DCM is recognized as the second most common type of genetically linked cardiac disease in the dog and is most prevalent in large or giant breeds (Dutton and López-Alvarez, 2018). Dobermans, Boxers, Great Danes, Newfoundlands, Irish Wolfhounds, English Cocker Spaniels, and Portuguese Water Dogs are the breeds with the highest prevalence of DCM (Monnet et al., 1995; Borgarelli et al., 2006; Werner et al., 2008; Martin et al., 2009). Moreover, Golden Retrievers and American Cocker Spaniels have recently emerged as being predisposed to taurine deficiency (Kramer et al., 1995; Bélanger et al., 2005). DCM is described as a primary myocardial disorder causing systolic dysfunction with secondary ventricular dilation, regular or decreased wall thickness, and increased cardiac mass due to myocyte enlargement (Maron et al., 2006). However, while pulse ingredients in grain-free diets have been suggested as causally linked to DCM, the actual link between them has not been definitively demonstrated to date (Kaplan et al., 2018; Mansilla et al., 2019b; McCauley et al., 2020). Despite an acknowledgment by the FDA in November 2020 that the link between DCM and diet in dogs is multi-factorial and not due solely to pulses, this issue remains unresolved, leaving veterinarians, and pet owners wary of pulse-containing dog foods.

The best evidence so far for a link between DCM and nutrition in dogs is through taurine insufficiency (Fascetti et al., 2003b; Backus et al., 2006b). Taurine is important to cardiac health because it participates in the reabsorption of calcium by the sarcoplasmic reticulum and enhances the sensitivity of the myofilaments to calcium (Bakker and Berg, 2002b). Therefore, as reviewed by Mansilla et al. (Mansilla et al., 2019b), because calcium is a key component of cardiac contraction, in cases of taurine absence due to reduced synthesis or low intake of taurine and/or its precursors, the cardiac muscle tissue is unable to properly contract and presumably develops DCM. Dog diets do not require taurine and taurine is not considered essential since dogs can synthesize taurine from sulfur-containing amino acids such as cysteine and methionine (Sanderson, 2006). Grain-free and pulse-containing diets are higher in fermentable fiber which has been demonstrated to decrease protein digestibility through either an increased fermentation of the sulfur-containing amino acids leading to decreased bioavailability or to higher fecal excretion (Kim et al., 1996c; Kim et al., 1996d). Previous studies have reported that high fermentable dietary fiber in dogs increases the need for dietary taurine or taurine precursors (e.g.,

methionine and cysteine) due to increased fecal excretion of taurocholate (the predominant bile acid in dogs), leading to fecal loss of taurine (E R O'Máille et al., 1965; Jon A. Story and Kritchevsky, 1978).

Fava beans have numerous anti-nutritional factors such as condensed tannins, trypsin inhibitor activity, lectins, and pyrimidine glucosides (LIENER, 1989).. Tannins are known to cause reduction of protein and energy digestibility (Rahate et al., 2021a). The pyrimidine glucosides (vicine and convicine) lead to favism which is a blood disorder caused when fava beans are eaten by humans with genetic mutations of G6PD, leading to decreased G6PD activity and reduced ability of red blood cells (RBCs) to produce ATP and regenerate glutathione (Luzzatto and Arese, 2018b). This then makes the red blood cell susceptible to oxidative damage, leading to rapid RBC death and acute anemia when uncooked fava beans are consumed. There are no reports of favism in dogs to the best of our knowledge. However, because of this concern, fava beans cannot be placed in the “generally assumed to be safe” category by AAFCO and thus is not currently used in pet food until proven otherwise.

Fermentation is a processing technique well-known for improving health, functional, and nutraceutical effects of foods (Frias et al., 2017). Fermentation also has been positively associated with enhanced nutritional quality of pulses by reducing their levels of anti-nutritional factors (Santana and Empis, 2001; Coda et al., 2010; Curiel et al., 2015). For example, fermentation of fava bean with *Lactobacillus plantarum* reduced the content of the anti-nutritional factors vicine and convicine by more than 90% while increasing the amount of free amino acids and enhancing protein digestibility (Coda et al., 2015b). Likewise, Rizzello et al. (Rizzello et al., 2016b) demonstrated a complete degradation of the pyrimidine glycosides in *L. plantarum*-fermented fava bean flour after 48 h, which indicates the usefulness of bioprocessing techniques for industrial fava bean detoxification. In comparison, some yeast organisms in the *Candida* clade have the potential to synthesize and increase taurine content while other yeasts and bacteria do not (Hébert et al., 2013a). This current study explored the use of fermentation with the yeast *Candida utilis* to both reduce anti-nutritional factors and increase taurine content.

The objective of this study was to determine if short-term (7-day) feeding of beagles with a moderate protein diet that has 30% inclusion of fava bean flour would show altered glucose tolerance, body weight, cardiovascular function, and blood parameters when contrasted to

commercial diets with normal vs. high protein. Two commercial diets with normal or high protein content and four different fava bean-containing diets with low- (LT) or high-tannin (HT) content were fed, both varieties with and without fermentation. We hypothesized that pulse-based diets would impair cardiovascular health due to the low taurine, cysteine, or methionine levels and high fiber content. Moreover, fermentation of fava bean flour with *C. utilis* would enhance diet quality and, consequently, health of dogs.

3.4 Materials and Methods

3.4.1 Fava bean ingredients and fermentation protocol

Low-tannin (Snowdrop) and HT (Florent) fava bean varieties, genotypes grown in Saskatchewan (Fleury et al.), were dehulled and ground into flour using a 400 µm screen. Fermentation of each variety was adapted from methodology used previously in pea flour in our laboratory (Curso Almeida, 2020). Briefly, *Candida utilis* (ATCC 9950) was maintained in sterile 80% (v/v) glycerol solution at -80°C, then reactivated on YGC agar plates when needed (Yeast Extract Glucose Chloramphenicol Agar; catalogue number 95765, Sigma Aldrich, St Louis, MO). Seeded plates were incubated at 30°C for 72 hours. Two loops of colonies were transferred using a flame-sterilized platinum needle to a 250 mL sterile conical flask containing 100 mL of YPD liquid medium (Yeast Peptone Dextrose- A1374501 ThermoFisher, Waltham, MA). The flask was incubated in a horizontal shaker incubator (30°C) at 120 rpm for 12 to 15 hours. After this period, 10 mL of the cultured yeast mass was transferred into a 500 mL sterile conical flask containing 250 mL of YPD liquid medium. The medium containing the yeast was then incubated on a horizontal shaker (30°C) at 120 rpm for an additional 12 to 15 hours. Twenty kilo batches of each fava bean variety were mixed with the yeast broth, ammonia and sterile water to form a soft dough, then fermented in an adapted cement mixer with temperature maintained at 30°C. The fermentation slurry was mixed for 3 minutes every hour for 72 hours. Samples were collected every 24 hours, and serial dilutions of mixture plated on sterile agar plates, then incubated at 30°C for 72 hours to verify yeast viability and count throughout the process. The fermented fava bean flour was subsequently dried in an oven (60°C) for 48h at the WCVF before transport to the University of Saskatchewan Canadian Feed Research Centre (North Battleford, Canada) for grinding of the fermented flours, followed by mixing of all test diets (both fermented and unfermented fava bean flours), extruding and vacuum coating with fat to produce the final dry kibble format of the diets to be used in the feeding trials.

3.4.2 Animals and diets

All animal use and procedures were approved by the University of Saskatchewan Animal Care Committee (Animal Utilization Protocol #20190055) and adhered to the Canadian Council on Animal Care. Eight neutered Beagles, four males and four females, at ideal body weight (8.8 ± 1.9 kg) and a mean age of 2.6 ± 1.0 years, were obtained from King Fisher International (Toronto, ON, Canada) or Marshall Bioresources (North York, NY, USA) and housed at the Western College of Veterinary Medicine (Saskatoon, SK, Canada). The animals were housed individually in 1.1×2.7 -m kennels with outdoor kennel access at night and during feeding but kept in a group kennel area during the day. Dogs were walked or socialized with volunteers for at least 1 hour every day. When not on trial, dogs were fed a commercial adult maintenance dry diet (Purina Dog Chow; Ralston Purina Co, St Louis, MI) in amounts that maintained each individual dog at ideal condition (score of 4-6 on a 9-point scale) in conjunction with energy requirements stated in the National Research Council guidelines (Council, 2003). All dogs were acclimated to procedures with rewards prior to experiments starting to minimize stress, thus no anesthetics or sedatives were used in this study.

In total, six diets were tested. Low- or high-tannin fava bean flours were used at 30% inclusion, in either fermented or unfermented formats with diet formulations indicated in Table 3.1. The 4 fava bean test diets were: Unfermented low-tannin (UF-LT; 27% crude protein (CP) DM (dry matter)), fermented low-tannin (FM-LT; 28% CP DM), unfermented high-tannin (UF-HT; 27% CP DM), fermented high-tannin (FM-HT; 28% CP DM). Insoluble ash (Celite™) was included at 1% as a non-digestible marker. Diets were formulated in accordance with the nutrient guidelines for adult dog maintenance set by AAFCO to be nutritionally balanced before being extruded under identical process conditions (Staff, 1998). The two additional diets tested were commercial diets containing normal-protein (NP; Purina Dog Chow™, St. Louis, MI; 24% CP DM) or high-protein content (HP; GO! Solutions Carnivore™, Richmond, BC; 41% CP DM). Ingredient lists for the commercial diets are shown in Table 3.2. All 6 diets were randomly sub-sampled and sent for proximate analysis (Central Testing, Winnipeg, MB, Canada) (Table 3.3). The analyzed content of crude fiber, non-fiber carbohydrates, metabolizable energy, vicine, and convicine of UF-LT, UF-HT, FM-LT, and FM-HT diets are 1.1, 0.5, 0.5, and 0.5%, 60.7, 62.1, 61.6, and 61.8%, 3.7, 3.8, 3.8, and 3.8 kcal/g, 1.8, 1.8, 0.4, and 0.6 mg/g, and 0.5, 0.6, 0.1, and 0.2 mg/g, respectively. The analyzed content of crude fiber, non-fiber carbohydrates, and

metabolizable energy of NP and HP diets are 0.8 and 1.1%, 53.0 and 29.8%, and 4.0 and 4.1 kcal/g, respectively. Dogs were fed each diet for 7 days, with the NP or HP diets fed during the first and sixth weeks, respectively. From week 2 to 5, using a randomized, crossover, 2×2 Latin square design, the UF-LT, FM-LT, UF-HT, and FM-HT diets were fed. Commercial diets were not included in the crossover design because the fava bean-based diets were formulated to contain the same nutrient profile to enable a reasonable comparison. In contrast, NP and HP diets have different ingredients at unknown inclusion levels (specific formulation not available on label), making direct comparisons difficult. Dogs were weighed at the beginning of the experiment and after each feeding week. The amount of diet allotted per dog per day was calculated to be isocaloric with the daily requirement for that dog using standard equations that determine the energy requirements for individual dog maintenance [maintenance energy (ME in kcal) = $[(70 \times BW^{0.75}) \times 1.6]$, daily portions divided, and dogs fed twice daily (at 08:30 and 16:30 h). Bowls were removed before the next meal and any uneaten food was weighed and recorded. All dogs generally consumed all food portioned in each meal within 5-10 minutes, with no palatability issues noted.

Table 3.1. Diet formulation for lab-formulated fava bean diets (%).

Ingredient	Unfermented		Fermented	
	Low tannin	High tannin	Low tannin	High tannin
Wheat flour	53.28	53.20	53.28	53.20
Low tannin fava bean fermented	-	-	30.00	-
Low tannin fava bean unfermented	30.00	-	-	-
High tannin fava bean fermented	-	-	-	30.00
High tannin fava bean unfermented	-	30.00	-	-
Turkey meal	11.74	13.04	11.74	13.04
Canola oil	1.40	1.00	1.40	1.00
Celite TM	1.00	1.00	1.00	1.00
Vitamin mixture	1.00	1.00	1.00	1.00
Mineral mixture	0.10	0.10	0.10	0.10
Salt	0.30	0.30	0.30	0.30
Dicalcium phosphate	0.53	0.26	0.53	0.26
Calcium carbonate	0.55	-	0.55	-
Choline chloride	0.10	0.10	0.10	0.10

Table 3.2. Ingredient composition of normal and high protein commercial diets. Ingredients are listed in order of decreasing inclusion.

Normal protein commercial diet
Whole grain corn, meat and bone meal, corn gluten meal, beef fat naturally preserved with mixed-tocopherols, soybean meal, poultry by-product meal, chicken, egg and chicken flavor, whole grain wheat, animal digest, salt, calcium carbonate, potassium chloride, mono and dicalcium phosphate, L-Lysine monohydrochloride, choline chloride, zinc sulfate, ferrous sulfate, manganese sulfate, copper sulfate, calcium iodate, sodium selenite, Vitamin E supplement, niacin (Vitamin B-3), Vitamin A supplement, calcium pantothenate (Vitamin B-5), pyridoxine hydrochloride (Vitamin B-6), Vitamin B-12 supplement, thiamine mononitrate (Vitamin B-1), Vitamin D-3 supplement, riboflavin supplement (Vitamin B-2), menadione sodium bisulfite complex (Vitamin K), folic acid (Vitamin B-9), biotin (Vitamin B-7), Yellow 6, Yellow 5, Red 40, Blue 2, garlic oil.
High protein commercial diet
Chicken Meal, Turkey Meal, Salmon Meal, De-Boned Chicken, De-Boned Turkey, De-Boned Trout, Potatoes, Chicken Fat (Preserved With Mixed Tocopherols), Peas, Tapioca, Lentils, Duck Meal, Chickpeas, Natural Chicken Flavor, Whole Dried Egg, Apples, Herring Meal, Flaxseed, Salmon Oil, Alfalfa, De-Boned Duck, De-Boned Salmon, Sweet Potatoes, Potassium Chloride, Pumpkin, Carrots, Bananas, Blueberries, Cranberries, Broccoli, Blackberries, Squash, Papayas, Pomegranate, Dried Chicory Root, Dried Lactobacillus Acidophilus Fermentation Product, Dried Enterococcus Faecium Fermentation Product, Dried Aspergillus Oryzae Fermentation Extract, Dried Bacillus Subtilis Fermentation Extract, Choline Chloride, Vitamins (Vitamin A Supplement, Vitamin D3 Supplement, Vitamin E Supplement, Niacin, L-Ascorbyl-2-Polyphosphate (A Source Of Vitamin C), D-Calcium Pantothenate, Thiamine Mononitrate, Beta-Carotene, Riboflavin, Pyridoxine Hydrochloride, Folic Acid, Biotin, Vitamin B12 Supplement), Minerals (Zinc Proteinate, Iron Proteinate, Copper Proteinate, Zinc Oxide, Manganese Proteinate, Copper Sulphate, Ferrous Sulphate, Calcium Iodate, Manganous Oxide, Selenium Yeast), Sodium Chloride, Taurine, Yucca Schidigera Extract, Dried Rosemary, Green Tea Extract, Peppermint, Parsley, Rosehips, Zedoary, Dandelion, Chamomile, Ginger, Fennel, Turmeric, Juniper Berries, Licorice, Marigold Extract, Cardamom, Cloves.

Table 3.3. Proximate composition (% , dry matter basis) of lab-formulated fava bean diets compared to commercial diets.

Item	Unfermented		Fermented		Commercial	
	Low tannin	High tannin	Low tannin	High tannin	Normal protein	High protein
Moisture	9.53	9.11	7.80	9.10	7.70	5.84
Dry matter	90.47	90.89	92.20	90.90	92.30	94.16
Crude protein	27.42	27.25	27.94	27.98	24.64	40.73
Crude fiber	1.05	0.47	0.50	0.52	0.79	1.05
Fat	2.71	2.51	2.07	2.15	13.10	17.38
Ash	7.20	6.74	7.01	6.67	7.56	10.07
Cystine	0.35	0.63	0.48	0.57	0.62	0.15
Methionine	0.32	0.29	0.31	0.30	0.36	0.61
Taurine	0.06	0.06	0.06	0.06	0.04	0.23
Non-fiber carbohydrates	60.72	62.12	61.56	61.77	52.99	29.83
Total digestible nutrients	82.85	83.88	83.57	83.90	82.73	79.94
Metabolizable energy (kcal/g)	3.72	3.80	3.76	3.80	4.04	4.09
Vicine (mg/g)	1.76	1.77	0.36	0.61	-	-
Convicine (mg/g)	0.50	0.58	0.12	0.22	-	-

3.4.3 Digestibility protocol

After a 5-day-feeding period on each fava bean-based diet, feces were collected during the subsequent 2 days of each period (total of 7 days on each diet). Collected feces were labelled and frozen at -20°C until analysis. Feces were thawed, homogenized, and pooled by dog (i.e. two samples from each dog pooled per dietary treatment). Prior to laboratory testing, feces were dried in a forced air oven at 55°C for 72 h and ground in a cutting mill with a 1-mm sieve. Diets and feces were analyzed (Central Testing, Winnipeg, MB) according to AOAC standards (Official Methods of Analysis, 21st Edition (2019)) for dry matter by oven-drying the sample, non-fiber carbohydrates, crude protein applying the Kjeldahl method, and acid-hydrolyzed fat. Gross energy (GE) content of diets was determined using a bomb calorimeter. The equation below was used to calculate the apparent digestibility coefficients of dry matter, crude protein, non-fiber carbohydrates, fat, gross energy, methionine, cysteine, and taurine for each fava bean-based diet using Celite as a nondigestible marker (Zhang and Adeola, 2017):

$$\text{Digestibility (\%)} = 100 - [100 \times (M_{\text{feed}} \times C_{\text{feces}} / M_{\text{feces}} \times C_{\text{feed}})]$$

Where M feed and M feces represent concentrations of index compound in feed and feces, respectively; C feed and C feces represent concentrations of components of interest in feed and feces, respectively.

3.4.4 Oral glucose tolerance test and blood analysis

After 7 days of feeding each diet, dogs were fasted for eight hours, then a syringe with 10 mL/kg body weight of a 10% glucose solution (1 g/kg BW glucose) fed to each dog by placing in the back of the mouth for an oral glucose tolerance test conducted at the same time each day. Prior to glucose feeding, the fasted dogs were aseptically catheterized using a peripheral intravenous catheter equipped with an extension tube inserted into the cephalic vein. Blood samples (~0.2 mL) were taken before feeding glucose (time 0) and at 15, 30, 60, and 90 min after feeding. Blood glucose was measured using a glucometer (OneTouch® Ultra 2™, LifeScan Inc, Johnson & Johnson, New Brunswick, NJ) with a minimum of duplicate readings for each time or until two consistent readings were obtained. The extension tube was filled with blood and initial blood discarded prior to glucometer reading to ensure no contamination of blood with anticoagulant solution. Subsequently, after each blood sample was obtained, the catheter was flushed with a sterile citrate solution to prevent clotting. The trapezoidal method was used to

determine the incremental area under the curve (AUC) for the glucose response (Wolever et al., 1991). Peak concentration and time to peak concentration for glucose were also calculated.

Additional fasting blood samples (8 mL) were taken using collection tubes with and without EDTA. Complete blood cell count (red (RBC) and white (WBC) blood cell counts) and chemistry panel (Cholesterol; Total (TB), Direct (DB), and Indirect Bilirubin (IB); Alkaline Phosphatase (ALP); Alanine Aminotransferase (ALT); Creatine Kinase (CK); Gamma-glutamyltransferase (GGT); Glutamate dehydrogenase (GLDH); total protein, albumin (A), globulin (G), and A:G) were analyzed at Prairie Diagnostic Services (Saskatoon, SK). Moreover, 3-mL subsamples of EDTA-tube blood from fasted animals were centrifuged at 2000 rpm for 10 minutes for plasma collection. Plasma samples were kept at -80°C until further analyses of methionine, cystine, cysteine, and taurine content (UC Davis Amino Acid Lab, Davis, CA). Plasma amino acid concentrations were analyzed in an automated amino acid analyzer via cation-exchange high-pressure liquid chromatography separation and ninhydrin-reactive colorimetric detection (Delaney et al., 2003a; Spitze et al., 2003b; Tôrres et al., 2003; Heinze et al., 2009).

3.4.5 Cardiac function, blood pressure and vascular health

After 7 days of feeding each diet, dogs were tested for cardiovascular health. All ultrasound measurements were performed and analysed by one individual. Before ultrasound, blood pressure was taken using a high-definition canine/feline oscillometer (VET HDO® High Definition Oscillometer, Babenhausen, Germany). An average of two readings with good agreement was used to determine diastolic and systolic pressures. Endpoints of flow-mediated dilation were used as an indicator of vascular health and included brachial artery diameter during baseline, during inflation of a blood pressure cuff placed distal to the brachial artery, and at the time of peak dilation (30 seconds) after cuff release, as previously determined by our research group in dogs (Raitakari and Celermajer, 2000; Adolphe et al., 2012). Echocardiography endpoints were used to assess cardiac function included heart rate (HR), stroke volume (SV) and cardiac output (CO) (Adolphe et al., 2012; Otto et al., 2019). Moreover, Left Ventricular End-diastolic volume (EDV), Left Ventricular End-systolic volume (ESV), Ejection fraction (EF), Left ventricular diastolic free wall thickness (DWT), Left ventricle systolic free wall thickness (SWT), Systolic blood pressure (SBP), Diastolic blood pressure (DBP), Velocity time integral

for bloodflow through the mitral valve (VTI), Maximum velocity of bloodflow through the mitral valve (MV) were also obtained. Flow-mediated dilation and echocardiography were measured using a SonoSite Edge II ultrasound (Fujifilm SonoSite inc., Bothell, WA) with detection using a P10x transducer (8-4 Hz) to detect cardiac endpoints and the L38xi (10-5 Hz) transducer for vascular imaging. Flow-mediated dilation was calculated using the following equation:

$$\%FMD = 100\% \times [(maximum\ diameter\ post-cuff\ release) - (baseline\ diameter)] / (baseline\ diameter).$$

Two-dimensional ultrasonography was used to measure left ventricular volume using the left parasternal apical two- and four-chamber views in diastole and systole (Lang et al., 2005). Two-dimensional guided M-mode echocardiography was used to obtain a right parasternal short-axis view of the heart at the level of the papillary muscles (Lang et al., 2005). Measurements of left ventricular end diastolic diameter (LVID_d) and left ventricular end systolic diameter (LVID_s) were also compiled from all dogs and normalized to body weight according to methodology described by Cornell et al. (Cornell et al., 2004).

3.4.6 Statistical analysis

Analyses were performed using SAS (version 9.4, SAS Institute Inc., Cary, NC). Before performing all analyses, the data was explored for normality and outliers using the PROC UNIVARIATE model in SAS and the Shapiro-Wilk test. One-way analysis of variance was used to compare differences among diets among normal vs high protein commercial diets and two-way analysis of variance was used to compare parameters for fava bean-based diets (fixed effects being fava bean variety and fermentation). In the present study, we used mixed-sex dogs in order to control the variation possibly associated with this factor. Previous studies from our group have not detected sex-related differences among (spayed/neutered) dogs (44) and sex-related differences are not related to the purposes of the present study, we did not include this factor in the model. All post hoc analyses were performed using the Fisher least significant difference (LSD) method. Differences were considered significant at $P < 0.05$.

3.5 Results

3.5.1 Body weight, meal portion, and body condition score

Body weight (BW), BCS and meal portion data are presented in Table 3.4. No significant effect ($P > 0.05$) of dietary protein content (NP vs HP) was observed on BCS or BW after 7 days of feeding in beagles. Within commercial diets, meal portion (163-187 g/day) was significantly smaller ($P < 0.05$) in HP compared to NP-fed dogs in order to restrict meal size to an isocaloric amount among diets. Within fava bean-based diets, there was no effect of either FM, FB, or the interaction between FM and FB on body weight, BCS or meal portion ($P > 0.10$).

Table 3.4. Body weight, food portion, and body condition score (BCS) of dogs fed diets formulated with either low or high tannin fava beans without (UF) or with (FM) fermentation, or normal (NP) vs high (HP) protein commercial diets for 7 days each*.

Item	Commercial			Low tannin		High tannin		SEM	P value ¹		
	NP	HP	SEM	UF	FM	UF	FM		P	FB	FM
Body weight (kg)	8.45	8.32	0.88	8.31	8.32	8.33	8.28	0.88	0.16	0.88	0.66
Food portion (g/d)	188	163	22.12	188	188	188	188	23.59	<0.01	1.00	1.00
BCS	4.62	4.50	0.18	4.56	4.62	4.55	4.50	0.18	0.35	0.34	0.95

* Eight mixed-gender, neutered beagles were fed the NP or HP diets during the first and sixth weeks, respectively. From week 2 to 5, using a randomized, crossover, 2×2 Latin square design, 4 diets differing in fava bean variety and fermentation were compared as follows: unfermented (UF) high tannin, fermented (FM) high tannin, UF low tannin and FM low tannin. Values expressed as means ($n = 8$). SEM = Pooled standard error of the mean.

¹P = P-value between commercial diets with different protein content (NP vs HP), FB = Fava bean (Low tannin vs High tannin), FM = Fermentation (UF vs FM). The interaction between FB and FM was not significant for any of the parameters measured ($P > 0.10$).

3.5.2 Glucose tolerance

Time course of blood glucose responses to the oral glucose tolerance test are shown in Figure 3.1 with fasting and peak glucose levels (mmol/L), time to peak (min), and area under the curve (mmol/L x min) data shown in Table 3.5. There was no effect ($P > 0.10$) of dietary protein content (NP vs HP) and no effect of FM, FB, or the interaction between FM and FB on fasting and peak glucose levels, time to peak or AUC.

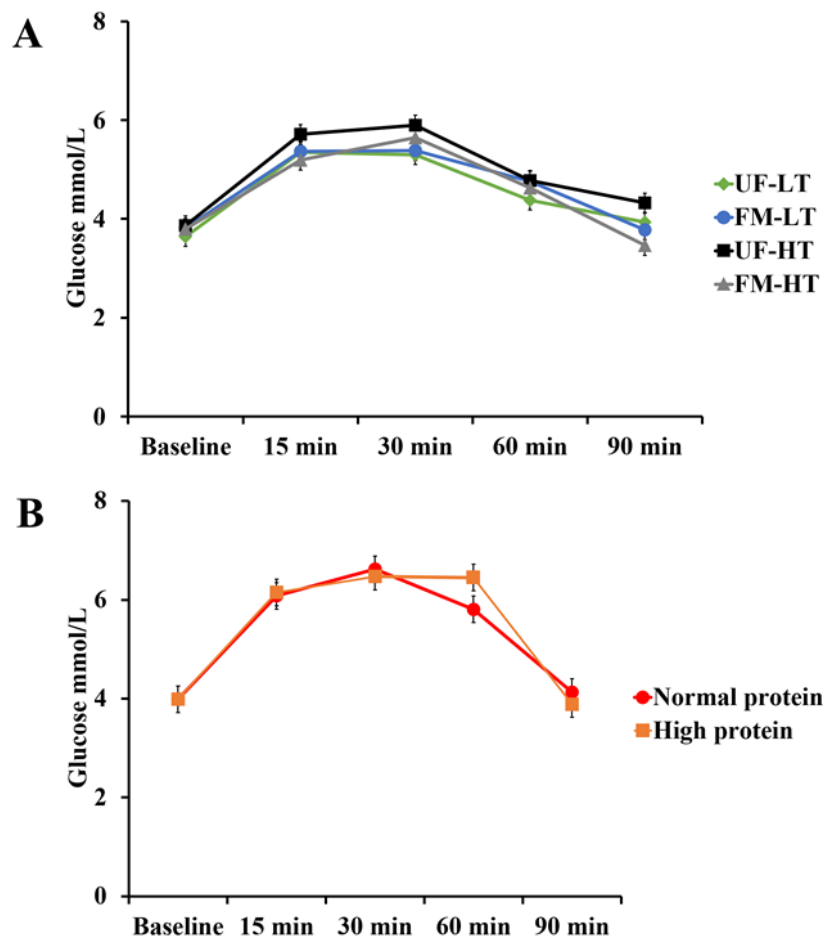


Figure 3.1. Blood glucose responses to the oral glucose tolerance test in dogs fed either low (LT) or high (HT) tannin fava bean–based diets without (UF) or with (FM) fermentation (A), or normal vs. high protein commercial diets (B). Eight mixed-gender, neutered beagles were fed the NP, or HP diets during the first and sixth weeks, respectively. From weeks 2 to 5, using a randomized, crossover, 2×2 Latin square design, 4 diets differing in fava bean variety and fermentation were compared as follows: UF-HT, FM-HT, UF-LT, and FM-LT. Fasting and peak glucose levels, time to peak, and area under the curve (AUC) were not influenced by dietary treatments ($P > 0.10$).

Table 3.5. Fasting and peak blood glucose levels, time to peak and area under the curve of dogs fed diets formulated with either low or high tannin fava beans without (UF) or with (FM) fermentation, or normal (NP) vs high (HP) protein commercial diets for 7 days each*.

Item	Commercial			Low tannin		High tannin		SEM	P value ¹		
	NP	HP	SEM	UF	FM	UF	FM		P	FB	FM
Fasting blood glucose level (mmol/L)	3.98	3.99	0.19	3.62	3.87	3.77	3.74	0.16	0.97	0.96	0.37
Peak blood glucose level (mmol/L)	7.02	6.96	0.35	5.69	5.81	6.33	5.80	0.23	0.92	0.15	0.36
Time to peak (min)	37.50	41.25	7.15	31.53	30.09	26.26	32.31	5.56	0.76	0.78	0.68
Area under the curve (mmol/L x min)	321.98	324.38	15.50	273.59	275.07	282.47	265.02	9.26	0.91	0.95	0.41

* Eight mixed-gender, neutered beagles were fed the NP or HP diets during the first and sixth weeks, respectively. From week 2 to 5, using a randomized, crossover, 2 × 2 Latin square design, 4 diets differing in fava bean variety and fermentation were compared as follows: unfermented (UF) high tannin, fermented (FM) high tannin, UF low tannin and FM low tannin. Values expressed as means (n = 8). SEM = Pooled standard error of the mean.

¹P = P-value between commercial diets with different protein content (NP vs HP), FB = Fava bean (Low tannin vs High tannin), FM = Fermentation (UF vs FM). The interaction between FB and FM was not significant for any of the parameters measured (P > 0.10).

3.5.3 Red and white blood cell count

White blood cell and RBC of beagles after feeding each diet for 7 days are shown in Figure 3.2. Dogs fed HP diets showed decreased RBC compared to NP-fed dogs ($P < 0.05$). Conversely, WBC tended to increase in dogs fed HP diets compared to NP-fed dogs ($P < 0.10$). Within fava bean-based diets, FM increased RBC regardless of FB ($P < 0.05$) and no effect ($P > 0.10$) of either FM, FB, or the interaction between FM and FB was observed on WBC.

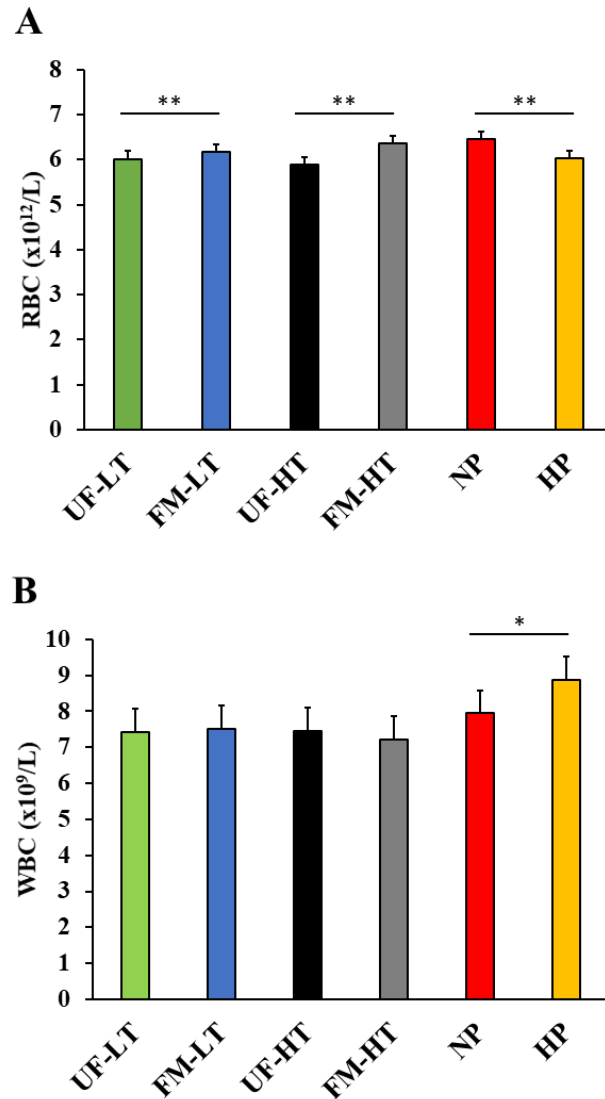


Figure 3.2. Red [RBC; (A)] and white [WBC; (B)] blood cell counts in dogs fed either low (LT) or high (HT) tannin fava bean-based diets without (UF) or with (FM) fermentation, or normal vs. high protein commercial diets. Eight mixed-gender, neutered beagles were fed the NP, or HP diets during the first and sixth weeks, respectively. From weeks 2 to 5, using a randomized, crossover, 2×2 Latin square design, 4 diets differing in fava bean variety and fermentation were compared as follows: UF-HT, FM-HT, UF-LT, and FM-LT * $P < 0.10$; ** $P < 0.05$.

3.5.4 Blood parameters of hepatic function

Blood parameters indicative of hepatic function in beagles after feeding test diets for 7 days are shown in Table 3.6. Within commercial diets, serum cholesterol was increased, while bilirubin parameters (TB, DB, and IB) were decreased in dogs fed HP diets compared to NP-fed dogs ($P < 0.05$). Furthermore, serum ALP and ALT were decreased in dogs fed HP diets compared to NP-fed dogs ($P < 0.05$). Moreover, total serum protein was decreased in dogs fed HP diets compared to NP-fed dogs, primarily due to a decrease in serum globulin, which elevated the albumin:globulin ratio ($P < 0.05$). There was no effect of dietary protein content on GGT, GLDH, CK, and albumin ($P > 0.10$). Among fava bean-based diets, DB was higher in dogs fed LT compared to HT-fed dogs regardless of FM ($P < 0.05$). There was no significant effect ($P > 0.10$) of either FM, FB, or the interaction between FM and FB on any other blood parameter of hepatic function measured.

Table 3.6. Blood parameters of hepatic function in dogs fed diets formulated with either low or high tannin fava beans without (UF) or with (FM) fermentation, or normal (NP) vs high (HP) protein commercial diets for 7 days each*.

Item	Commercial		SEM	Low tannin		High tannin		SEM	P value ¹		
	NP	HP		UF	FM	UF	FM		P	FB	FM
Cholesterol (mmol/L)	4.17	4.41	0.36	3.00	3.14	3.05	3.01	0.26	0.03	0.61	0.50
TB (μmol/L)	0.76	0.27	0.10	0.67	0.62	0.57	0.53	0.16	<0.01	0.55	0.78
DB (μmol/L)	0.43	0.18	0.07	0.37	0.38	0.28	0.21	0.08	0.01	0.05	0.65
IB (μmol/L)	0.33	0.09	0.07	0.30	0.23	0.20	0.32	0.12	0.02	0.96	0.80
ALP (U/L)	62.87	51.12	10.53	70.62	70.87	74.87	68.25	11.69	0.02	0.80	0.34
GGT (U/L)	0.37	0.68	0.30	2.25	1.62	1.75	2.37	0.74	0.31	0.84	1.00
ALT (U/L)	24.87	20.12	1.23	28.50	29.75	28.37	26.75	3.13	<0.01	0.27	0.89
GLDH (U/L)	4.75	4.50	0.45	5.25	6.12	5.12	4.50	1.22	0.51	0.28	0.87
CK (U/L)	148.25	197.37	19.65	116.27	143.32	165.23	163.52	17.73	0.06	0.10	0.51
Total protein (g/L)	52.75	51.62	1.65	50.75	51.00	51.12	51.37	1.49	0.03	0.80	0.86
Albumin (A; g/L)	31.75	32.75	1.36	32.12	32.25	32.25	32.25	1.32	0.06	0.89	0.89
Globulin (G; g/L)	21.00	18.87	0.76	18.62	18.75	18.75	19.12	0.62	<0.01	0.41	0.62
A:G	1.52	1.75	0.08	1.73	1.74	1.71	1.69	0.08	<0.01	0.52	0.85

*Eight mixed-gender, neutered beagles were fed the NP or HP diets during the first and sixth weeks, respectively. From week 2 to 5, using a randomized, crossover, 2 × 2 Latin square design, 4 diets differing in fava bean variety and fermentation were compared as follows: unfermented (UF) high tannin, fermented (FM) high tannin, UF low tannin and FM low tannin. Values expressed as means (n = 8). SEM = Pooled standard error of the mean. TB = Total bilirubin, DB = Direct bilirubin, IB = Indirect bilirubin, ALP = Alkaline phosphatase, GGT = Gamma-glutamyl transferase, ALT = Alanine aminotransferase, GLDH = Glutamate dehydrogenase, CK = Creatine kinase.

¹P = P-value between commercial diets with different protein content (NP vs HP), FB = Fava bean (Low tannin vs High tannin), FM = Fermentation (UF vs FM).

3.5.5 Blood electrolytes

Blood electrolytes from beagles after feeding each test diet for 7 days are shown in Table 3.7. Within commercial diets, serum bicarbonate, Ca, and P were increased in dogs fed HP compared to NP-fed dogs ($P < 0.05$). There was no effect ($P > 0.10$) of serum protein content on Na, K, Na:K, Cl, anion gap, or Mg. Among fava bean-based diets, serum P was increased in dogs fed HT compared to LT-fed dogs, regardless of FM ($P < 0.05$). Furthermore, FM tended to increase serum P compared to UF, regardless of FB ($P < 0.05$). There was an interaction between FB and FM for serum K and Na:K ($P < 0.05$). Dogs fed FM-HT diets showed the highest serum K, UF-HT the lowest, with FM-LT and UF-LT being intermediate. Consequently, dogs fed UF-HT diets showed the highest serum Na:K, FM-HT the lowest, with FM-LT and UF-LT being intermediate. There was no significant effect ($P > 0.10$) of either FM, FB, or the interaction between FM and FB on serum Na, Cl, bicarbonate, anion gap, Ca, and Mg.

Table 3.7. Blood electrolytes (mmol/L) of dogs fed diets formulated with either low or high tannin fava beans without (UF) or with (FM) fermentation, or normal (NP) vs high (HP) protein commercial diets for 7 days each*.

Item	Commercial		SEM	Low tannin		High tannin		SEM	P value ¹			
	NP	HP		UF	FM	UF	FM		P	FB	FM	FB*FM
Na	146.50	147.25	0.54	146.34	145.67	146.62	146.26	0.55	0.24	0.36	0.28	0.71
K	4.80	4.87	0.07	4.65 ^{cd}	4.62 ^{cd}	4.56 ^d	4.74 ^c	0.06	0.52	0.70	0.13	0.01
Na:K	30.50	30.00	0.56	31.26 ^{cd}	31.49 ^{cd}	32.19 ^c	31.08 ^d	0.51	0.54	0.47	0.23	0.04
Cl	113.63	113.88	0.60	112.13	111.54	111.65	112.21	0.65	0.62	0.81	0.97	0.12
HCO ₃ -	19.12	20.50	0.38	20.28	19.22	19.92	20.03	0.63	0.04	0.73	0.48	0.38
Anion gap	18.62	17.87	0.47	18.52	19.64	19.53	19.02	0.92	0.26	0.83	0.73	0.35
Ca	2.44	2.49	0.03	2.45	2.46	2.47	2.45	0.03	0.01	0.70	0.37	0.22
P	1.49	1.67	0.03	1.34	1.47	1.48	1.53	0.04	<0.01	0.05	0.08	0.42
Mg	0.88	0.81	0.08	0.85	0.82	0.83	0.84	0.01	0.56	0.87	0.55	0.11

*Values expressed as means (n = 8). SEM = Standard error of the mean.

¹P = P-value between commercial diets with different protein content (NP vs HP), FB = Fava bean (Low tannin vs High tannin), FM = Fermentation (UF vs FM).

^{c,d}Means within a same row with no common superscript differ significantly (P < 0.05)

3.5.6 Blood urea and creatinine

Blood urea and creatinine of beagles after feeding each test diet for 7 days are shown in Figure 3.3. Serum urea was increased ($P < 0.05$) while creatinine tended to increase ($P < 0.10$) in dogs fed HP compared to NP-fed dogs. There was no significant effect ($P > 0.10$) of either FM, FB, or the interaction between FM and FB on serum urea and creatinine.

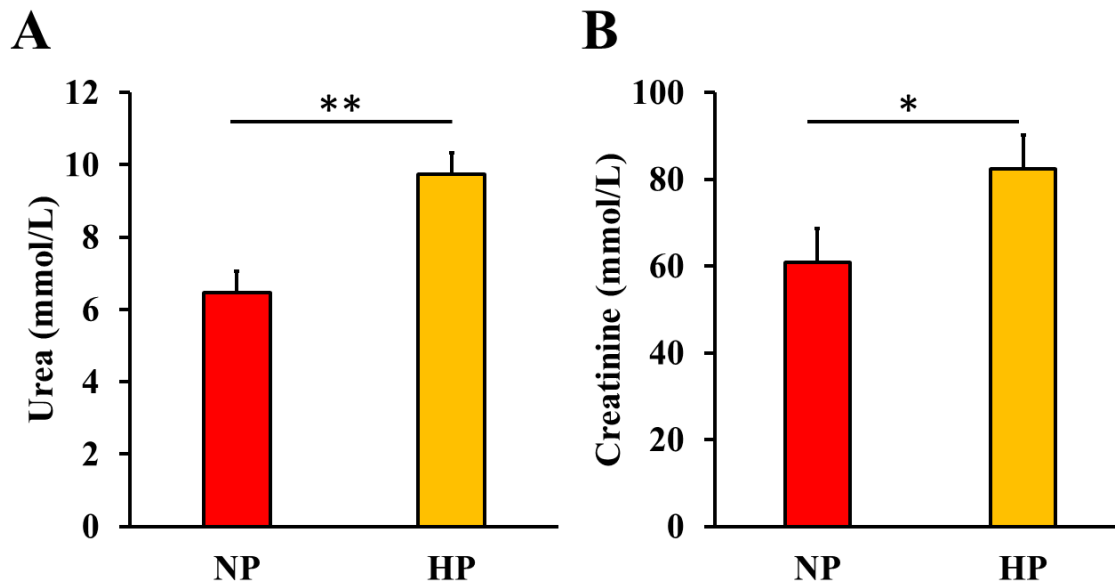


Figure 3.3. Blood urea (A) and creatinine (B) content in dogs fed either normal (NP) or high protein (HP) commercial diets. Eight mixed-gender, neutered beagles were fed the NP, or HP diets during the first and sixth weeks, respectively * $P < 0.10$; ** $P < 0.05$. There was no effect of either fava bean (FB), fermentation (FM), or the interaction between FB and FM on urea and creatinine content ($P > 0.05$).

3.5.7 Digestive enzymes in blood

After 7 days of feeding each test diet to dogs, there was an interaction between FB and FM, where serum amylase was highest in FM-HT, lowest in FM-LT, but intermediate in UF-LT and UF-HT ($P < 0.05$) (Figure 3.4). However, there was no significant effect of either FM or FB on serum amylase ($P > 0.10$). Moreover, dietary protein content did not significantly impact serum amylase ($P > 0.10$).

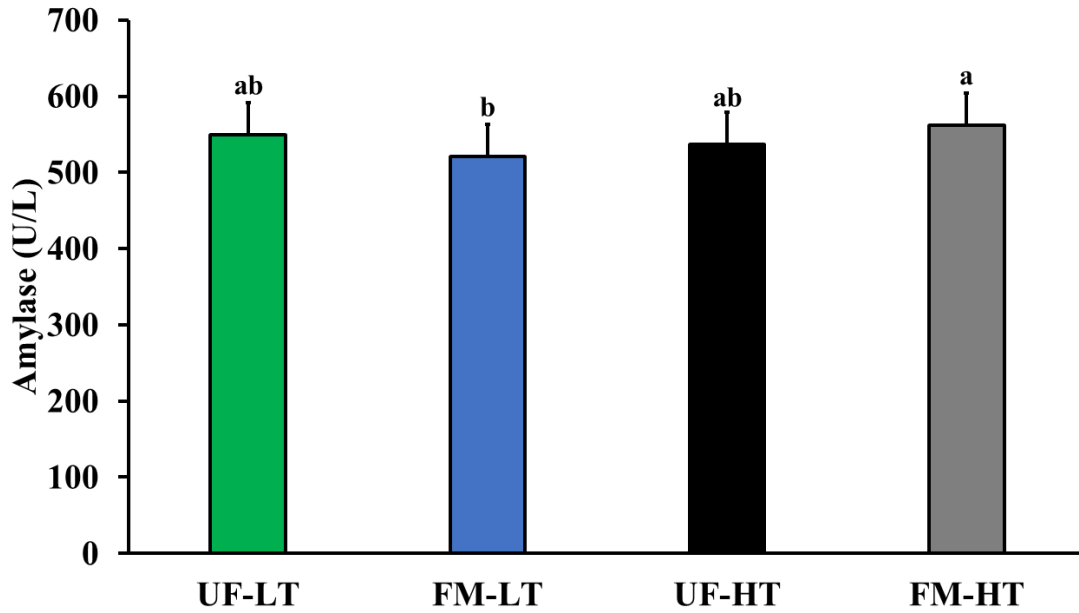


Figure 3.4. Blood amylase content in dogs fed either low (LT) or high (HT) tannin fava bean–based diets without (UN) or with (FM) fermentation. From weeks 2 to 5, using a randomized, crossover, 2×2 Latin square design, 8 mixed-gender, neutered beagles were fed 4 diets differing in fava bean variety and fermentation as follows: UF-HT, FM-HT, UF-LT, and FM-LT. Bars with no common letter (a, b) differ significantly ($P < 0.05$). There was no effect of dietary protein content on serum amylase content ($P > 0.05$).

3.5.8 Cardiovascular function

Cardiovascular function parameters of beagles after 7 days of feeding each test diet are shown in Table 3.8. Dogs fed HP had decreased left ventricular end-systolic volume (ESV) and cardiac output (CO) compared to NP-fed dogs ($P < 0.05$). Dogs fed FM diets tended to have decreased maximum velocity (MV) of passive ventricular filling (the early or E wave) compared to UF-fed dogs regardless of FB ($P < 0.10$). There was no significant effect of protein content or either FM, FB, or the interaction between FM and FB on left ventricular end-diastolic volume (EDV), stroke volume (SV), heart rate (HR), ejection fraction (EF), left ventricular diastolic wall thickness (DWT), left ventricular systolic wall thickness (SWT), systolic blood pressure (SBP), diastolic blood pressure (DBP), flow mediated dilation (FMD), velocity time integral for ventricular filling (VTI) (E and A wave combined), and MV (A wave) ($P > 0.10$). Moreover, there was no significant effect of dietary treatments on LVID_d and LVID_s ($P > 0.10$).

Table 3.8. Cardiovascular function parameters of dogs fed diets formulated with either low or high tannin fava beans without (UF) or with (FM) fermentation, or normal (NP) vs high (HP) protein commercial diets for 7 days each *.

Item	Commercial			Low tannin		High tannin		SEM	P value ¹		
	NP	HP	SEM	UF	FM	UF	FM		P	FB	FM
EDV (mL)	24.25	21.33	2.50	19.36	16.84	16.86	17.33	1.89	0.42	0.60	0.59
ESV (mL)	8.67	4.37	1.32	2.97	2.36	2.59	2.42	0.60	0.05	0.79	0.52
LVID _d (cm/BW ^{1/3})	0.96	0.88	0.07	0.92	0.85	0.83	0.90	0.08	0.43	0.85	0.99
LVID _s (cm/BW ^{1/3})	0.47	0.43	0.05	0.37	0.37	0.36	0.36	0.04	0.58	0.79	1.00
SV (mL)	18.62	16.96	1.37	16.39	14.59	14.29	14.91	1.41	0.41	0.53	0.68
CO (L/min)	1.84	1.41	0.14	1.41	1.34	1.41	1.21	0.18	0.04	0.72	0.44
EF (%)	79.38	83.13	3.49	85.88	86.44	85.56	87.31	3.20	0.46	0.89	0.60
HR (bpm)	97.75	93.13	5.05	102.83	91.17	101.29	91.50	5.55	0.53	0.91	0.07
DWT (cm)	0.83	0.79	0.07	0.85	0.89	0.90	0.88	0.05	0.72	0.57	0.90
SWT (cm)	1.52	1.35	0.13	1.50	1.37	1.48	1.39	0.08	0.38	0.99	0.17
SBP (mmHg)	136.12	128.93	5.52	131.31	118.06	127.75	123.25	7.95	0.37	0.91	0.27
DBP (mmHg)	76.88	72.57	6.58	67.42	61.87	63.80	62.50	5.57	0.65	0.79	0.54
FMD (%)	13.25	6.78	4.03	6.88	10.35	6.54	9.66	4.35	0.27	0.90	0.45
VTI (E wave) (cm)	9.87	9.23	2.14	7.49	8.43	9.08	8.97	1.23	0.83	0.40	0.74
VTI (A wave) (cm)	1.10	0.82	0.22	1.02	1.13	1.40	1.21	0.21	0.39	0.30	0.84
MV (E wave) (cm/s)	87.80	82.46	13.30	72.08	65.86	83.00	80.40	6.58	0.76	0.06	0.51
MV (A wave) (cm/s)	44.31	37.03	4.76	40.67	35.75	49.14	41.32	5.21	0.30	0.19	0.23

*Eight mixed-gender, neutered beagles were fed the NP or HP diets during the first and sixth weeks, respectively. From week 2 to 5, using a randomized, crossover, 2 × 2 Latin square design, 4 diets differing in fava bean variety and fermentation were compared as follows: unfermented (UF) high tannin, fermented (FM) high tannin, UF low tannin and FM low tannin. Values expressed as means (n = 8). SEM = Pooled standard error of the mean. EDV = Left Ventricular End-diastolic volume, ESV = Left Ventricular End-systolic volume; LVID_d = left ventricular end diastolic diameter, LVID_s = left ventricular end systolic diameter, SV = Stroke volume, HR = Heart rate, CO = Cardiac output, EF = Ejection fraction, DWT = Left ventricle diastolic wall thickness, SWT = Left ventricle systolic wall thickness, SBP = Systolic blood pressure, DBP = Diastolic blood pressure, FMD = Flow mediated dilation, VTI (E wave) = Velocity time integral (E wave), VTI (A wave) = Velocity time integral (A wave), MV (E wave) = Maximum velocity (E wave), MV (A wave) = Maximum velocity (A wave).¹P = P-value between commercial diets with different protein content (NP vs HP), FB = Fava bean (Low tannin vs High tannin), FM = Fermentation (UF vs FM). The interaction between FB and FM was not significant for any of the parameters measured.

3.5.9 Digestibility

Apparent total tract digestibility in beagles fed fava bean-based diets are shown in Table 3.9. Dogs fed FM diets had decreased fat digestibility and increased non-fiber carbohydrates digestibility compared to UF diets regardless of FB ($P < 0.05$). There was no significant effect of FM, FB, or the interaction between FM and FB on digestibility of crude protein, gross energy, methionine, cysteine, or taurine ($P > 0.10$).

Table 3.9. Apparent total tract digestibility of dogs fed diets formulated with either low or high tannin fava beans without (UF) or with (FM) fermentation for 7 days each*.

Item	Low tannin		High tannin		SEM	P value ¹	
	UF	FM	UF	FM		FB	FM
Crude protein	85.74	86.80	85.91	84.74	1.04	0.37	0.95
Fat	91.93	89.15	92.44	89.15	1.26	0.66	0.01
Non-fiber carbohydrates	95.94	96.59	96.09	96.64	0.21	0.76	0.05
Gross energy	92.02	92.19	91.85	91.61	0.35	0.29	0.91
Methionine	85.87	84.56	83.28	83.32	1.29	0.15	0.62
Cysteine	84.38	87.34	88.26	87.34	1.49	0.21	0.50
Taurine	86.68	84.08	84.88	84.87	1.55	0.74	0.41

*From week 2 to 5, using a randomized, crossover, 2×2 Latin square design, eight mixed-gender, neutered beagles were fed 4 diets differing in fava bean variety and fermentation as follows: unfermented (UF) high tannin, fermented (FM) high tannin, UF low tannin and FM low tannin. Values expressed as means ($n = 8$). SEM = Pooled standard error of the mean.

¹FB = Fava bean (Low tannin vs High tannin), FM = Fermentation (UF vs FM). The interaction between FB and FM was not significant for any of the parameters measured.

3.5.10 Plasma amino acid levels

Plasma amino acid concentration in beagles after 7 days of feeding each test diet are shown in Table 3.10. Dogs fed HP diets had increased taurine, cystine, and cysteine concentrations compared with NP-fed dogs ($P < 0.05$). There was no significant effect of dietary protein content on plasma methionine concentration ($P > 0.10$). Plasma cysteine was increased in HT-fed dogs compared to LT-fed dogs regardless of fermentation ($P < 0.05$). There was no significant effect of fava bean variety on plasma taurine, cystine, and methionine levels ($P > 0.05$). However, fermentation increased plasma cysteine ($P < 0.05$) and tended to decrease plasma cystine ($P < 0.10$) regardless of fava bean variety. There were no significant interactions between fava bean variety and fermentation on plasma amino acid levels ($P > 0.10$).

Table 3.10. Plasma amino acid levels (nmol/mL) of dogs fed diets formulated with either low or high tannin fava beans without (UF) or with (FM) fermentation, or normal (NP) vs high (HP) protein commercial diets for 7 days each*.

Item	Commercial			Low tannin		High tannin			P value ¹		
	NP	HP	SEM	UF	FM	UF	FM	SEM	P	FB	FM
Taurine	60.83	116.38	13.58	79.81	74.91	90.88	87.39	9.95	0.01	0.22	0.65
Cystine	10.83	13.33	0.77	16.75	14.75	16.38	13.75	1.47	0.04	0.59	0.08
Cysteine	54.00	472.50	20.40	85.75	137.25	112.62	153.87	35.86	<0.01	0.05	<0.01
Methionine	55.20	51.50	2.20	44.57	45.86	44.67	41.00	2.55	0.26	0.36	0.64

*Eight mixed-gender, neutered beagles were fed the NP or HP diets during the first and sixth weeks, respectively. From week 2 to 5, using a randomized, crossover, 2 × 2 Latin square design, 4 diets differing in fava bean variety and fermentation were compared as follows: unfermented (UF) high tannin, fermented (FM) high tannin, UF low tannin and FM low tannin. Values expressed as means (n = 8). SEM = Pooled standard error of the mean.

¹P = P-value between commercial diets with different protein content (NP vs HP), FB = Fava bean (Low tannin vs High tannin), FM = Fermentation (UF vs FM). The interaction between FB and FM was not significant for any of the parameters measured.

3.6 Discussion

The objective of this study was to determine if neutered, mixed-gender, adult beagles fed diets with 30% inclusion of fava bean flour would show altered nutrient digestibility, glucose tolerance, overall health, cardiovascular function, and plasma amino acid levels when contrasted to commercial diets with normal vs. high protein. Fava bean diets had moderate protein levels, but were formulated to be near the AAFCO dietary minimums for methionine (0.33% inclusion) or cystine + methionine (0.65% inclusion) (Staff, 1998). This was done intentionally to cause more rapid changes in sulfur-containing amino acid levels in the dogs and potentially cause early, reversible impairments in cardiac function despite using only a 7-day feeding period for each test diet. All fava bean diets in this study met the cystine + methionine AAFCO minimum, but all were slightly below the minimum for methionine alone due to variations in methionine content of the fava beans from reported literature values that were used for diet formulation (Frias et al., 2017).

3.6.1 Lack of Evidence for Toxicity From Fava Beans, Digestibility, Glucose Tolerance, and Anti-nutritional Factors

Fava beans are pulses, a subset of legumes. Other legumes such as peas have been increasingly included in dog diets as a protein and fiber source (Butterwick et al., 1994; Rice and Ihle, 1994). Pulse ingredients have been controversially associated with grain-free diets and the occurrence of DCM in dogs (Mansilla et al., 2019b). Specific to fava beans, however, is the additional association from vicine and convicine anti-nutritional factors with hemolytic anemia in susceptible humans (Champ, 2002). Worries about potential dog toxicity have prevented its AAFCO approval as a dog food ingredient thus far. Fermentation has been used as a valuable approach to reduce anti-nutritional factors in pulses, including trypsin inhibitors, hemagglutinins, and saponins. Moreover, *Candida* species have the potential to synthesize and increase taurine content (Hébert et al., 2013a) as well as improve protein digestibility through its breakdown into amino acids by fermentative microorganisms (Malcolmson and Han, 2019). This current study is the first to show that fermentation with *C. utilis* can successfully reduce the vicine/convicine content in fava bean flour, but had no effect on taurine content, protein digestibility, or amino acid digestibility. Of interest, however, is the observation that fermented fava bean diets both caused increases in plasma cysteine after 7 days of feeding, an effect that does not seem to relate to dietary levels and has no current explanation that should be explored in future studies.

The low-tannin variety used in the present study, Snowdrop, is known to have tannin levels as low as 1% (Wei, 2019), while Florent, which was the high tannin variety used in the present study, has been classified as a normal (higher) tannin genotype in Canada (Fleury et al.). Generally, other anti-nutritional factors tend to be high in varieties with high tannins, but both fava bean varieties used in the present study had high concentration of the anti-nutritional factors vicine and convicine. The main issue associated with high tannin content in the diet is related to reduced bioavailability of nutrients in the gastrointestinal tract (Addisu, 2016; Bunglavan and Dutta). Despite not evaluating bioavailability in the present study, there was no effect of fava bean variety on digestibility values of any nutrient measured. This implies that despite the potential negative effects of tannins on, specially, protein digestibility (C et al., 2011), we do not have evidence to show a clear detrimental effect of tannins. However, within-animal variation in digestive response to tannins and low sample size may have prevented detection of an impact on digestibility (A.e et al., 1992). Interestingly, fermentation was able to dramatically reduce both the vicine and convicine content in fava bean-based diets, regardless of variety. In a recent study, fermentation with *L. plantarum* degraded the pyrimidine glycosides in fava bean flour within 48 h, which reduced the toxicity of the fermented fava bean as assessed through *ex vivo* assays on human blood (Rizzello et al., 2016b). In the present study, there was no impairment in glucose tolerance in dogs fed fermented fava bean (FM) diets as revealed by the lack of effect on glucose baseline and peak levels, time to peak, and AUC. This leads to the conclusion either that fava beans do not have any effect on glucose utilization in dogs or that 7 days is not sufficient to alter glucose utilization. However, it should be noted that the four fava bean test diets in this study were fed sequentially in a crossover design. By the end of these four feeding periods, beagles had been fed fava bean-based diets for a month, with no change in glucose utilization from the previous NP period. Moreover, RBC content was unchanged in dogs fed fava beans compared with the NP diet. Taken together, because anemia is a primary sign of favism and none of the fava bean-based diets caused anemia in the dogs in the current study, it can be concluded that fava beans are not toxic in dogs. While AAFCO requires a 6-month feeding study for fava beans to be approved as a pet food ingredient, this study provides initial indications that they are a safe dog food ingredient.

The present study also provided other indication of fermentation of fava bean flour with *C. utilis* enhancing diet quality and consequently health in dogs. Fermentation significantly

decreased the digestibility of fat and increased the digestibility of non-fiber carbohydrates. It has been shown in fish that the concentration of carbohydrates in the gut are inversely related to fat digestibility (Storebakken et al., 1998) and this is exacerbated when large amounts of starch are present (Skrede et al., 2002). Surprisingly, amylase content in blood was increased in FM-HT compared with FM-LT with both unfermented diets being intermediate. This indicates that the fermentability of fava bean varieties may be different, which might be associated with the carbohydrate composition, releasing increased or decreased amounts of starch when fermented (Çalışkantürk Karataş et al., 2017). Finally, dogs fed fermented diets showed increased RBC levels compared with those fed unfermented fava bean diets. Low RBC in young dogs is a common occurrence as RBC lifespan is shorter and young RBC contain less hemoglobin when compared with aging RBC (Bush, 1991). An increased RBC content in fermented diet-fed dogs could be associated with an accelerated production of blood cells and increased health that should be further explored in future studies.

3.6.2 Effect of High Dietary Protein Diet on Dog Health

The results of the current study using commercial diets also provide indications that high dietary protein can negatively affect overall health of dogs. Previous studies have reported that high protein dog diets may negatively impact gastrointestinal health, pre-disposing dogs to diarrhea ([61](#)). While diarrhea was not observed with the HP commercial diet tested in the current study, excessive protein intake has been reported to increase proteinuria and overload kidneys, potentially decreasing the overall health of dogs ([62](#)), and this is consistent with the higher serum cholesterol, urea, and creatinine observed with the HP diet in the current study. High serum cholesterol in dogs is consistent with a positive relationship reported between protein intake and cholesterol in humans ([63](#)). Moreover, bilirubin measurements (TB, DB, and IB) were decreased in HP-fed dogs compared with NP, which agrees with a recent study showing higher bilirubin concentrations in young pigs fed a protein restricted diet compared with a control diet ([64](#)). On the other hand, potential benefits of the HP diet in the current study come from the observed decrease in ALP and ALT in dogs fed HP compared with NP. In dogs, higher ALT content is directly associated with hepatocyte membrane damage and necrosis, whereas ALP is positively associated with biliary stasis ([65](#), [66](#)). Increased ALT and ALP in NP dogs may be interpreted as poor hepatic function ([67](#)). It should be noted that while trends for changes in blood parameters can be interpreted as positive or negative, all values for all end-points measured in this study fell

within clinical norms and thus all dogs were maintained in a healthy state. Future studies using longer feeding periods are required to determine if trends continue and become clinically significant compared to that observed after 7 days in the current study.

3.6.3 Cardiac Function, Cysteine, Methionine, and Taurine

One of the main hypotheses of the present study was that pulse-based diets with their higher fiber would cause decreases in plasma taurine, cysteine, or methionine levels, subsequently leading to impaired cardiac contractility or enlargement of the heart consistent with DCM. However, after 7 days of feeding each fava bean–based diet, no significant adverse changes were detected in cardiac or vascular function in the current study. Longer feeding trials are needed to confirm that there are no effects. However, what did change in the short term was ESV that was increased in NP-fed dogs compared with HP-fed dogs, but without changes in ventricular chamber size (LVID). Changes in cardiac chamber size would be surprising after only 7 days, thus a functional change such as impaired contractility and reduce cardiac output (CO) is not expected to explain the higher ESV. In fact, the NP-fed dogs instead had higher CO that may be due to a non-significant trend for both HR and SV to increase. This is consistent with a similar non-significant trend for both systolic and diastolic blood pressure to increase in NP-fed dogs compared with HP-fed dogs, suggesting a generalized increase in sympathetic outflow in the NP-fed dogs. Higher sympathetic tone and blood pressure increases afterload and impairs emptying of the left ventricle at the end of systole (i.e., higher ESV), a reliable indicator of impaired systolic function (Kittleson and Kienle, 1998).

The proximate analysis of diets showed no major differences in crude fiber content between laboratory-formulated fava bean–based diets and commercial diets. The main differences among diets were observed for cysteine content, which was low in the fava bean diets. Also, methionine and taurine content were highest in HP diets compared with all fava bean–based diets and the NP diet. If reduced dietary taurine levels were driving cardiac changes, then it would have been expected that the NP diet should have led to adverse cardiac changes and this may agree with what we observed. Future studies should explore longer feeding periods and address whether more susceptible dog breeds than beagles produce a stronger relationship between taurine and cardiac impairment.

The fava bean–based diets and the NP commercial diet tested in the current study all had methionine content that was just below the AAFCO minimum. Dogs can synthesize taurine from cysteine or methionine (Harrison et al., 2020a) but all three of these amino acids tend to be low or limiting when plant-based protein sources such as pulses are used. Compounding this problem, pulses are high in resistant starch and fiber. Previous studies in dogs have reported that high fiber diets decrease protein digestibility and increase fecal bile acid excretion in feces (Pezzali et al., 2020a). Because taurocholate is the major bile salt excreted by dogs, the net effect of high fiber has been reported to deplete taurine and impair digestion of protein that contains cysteine and methionine needed to replace it. However, none of the diets tested caused any significant drop in plasma levels of taurine, cysteine, or methionine and levels remained above the reference range throughout the study (Delaney et al., 2003b). In fact, fermented fava beans (both varieties) led to significant increases in plasma cysteine, suggesting a potentially beneficial health effect of fermentation processing. In contrast, the high protein (HP) commercial diet led to higher plasma levels of cysteine and taurine, but no change in plasma methionine compared with NP diet, suggesting that 7 days was sufficient time to cause some alterations of blood levels of these amino acids. Cysteine results, however, should be cautiously interpreted due to its unstable nature and an interfering substance during HPLC analysis of this amino acid that could have led to overestimation of cysteine levels (Determination of free and total cyst(e)ine in plasma of dogs and cats - Tôrres - 2004 - Veterinary Clinical Pathology - Wiley Online Library). Further analysis using a different method would be necessary to confirm the cysteine results.

3.7 Strength and limitations

A strength of this study was that the fava bean–based diets that were made in our laboratory were tested against two popular commercial brands, giving a more realistic context to the results. Another strength was the use of a Latin square, crossover design where the same dogs were tested on each diet, reducing variability and increasing power with the small sample size of this experiment. However, an important limitation of this study was the duration, which is insufficient to cause major structural cardiac changes, but was long enough to change plasma levels of sulfur-containing amino acid levels, at least in response to high dietary protein. Moreover, the results of this study using young, healthy adult beagles may not apply to older, large breed dogs with genetic susceptibility to taurine deficiency. However, we would predict

that changes in taurine and sulfur-containing amino acids would be even more pronounced in these breeds or in older dogs. A last limitation of the present study was the experimental design which does not allow the statistical comparison between commercial and fava bean-based diets.

3.8 Conclusion and Implications

Most importantly, fava bean–based diets did not cause hemolytic anemia and did not alter glucose handling in dogs after 7 days of feeding, thus fava beans appear safe as a dog food ingredient. In contrast, the high-protein grain-free commercial diet adversely altered blood chemistry compared with the normal protein, grain-containing commercial diet we tested. Moreover, the normal protein, grain-based diet appeared to cause excess sympathetic tone, a trend that if it were to continue with long-term feeding, might lead to adverse changes in cardiac health that are distinct from DCM. On the other hand, fermentation with *C. utilis* looks promising to reduce anti-nutritional factors and potentially improve health through improvements in nutrient digestibility and increased RBC levels in dogs. Studies using longer feeding periods are needed to determine whether these short-term changes are sustained to produce clinically significant changes in dogs.

CHAPTER 4

THE EFFECTS OF FERMENTATION OF LOW OR HIGH TANNIN FAVA BEAN-BASED DIETS ON GLUCOSE RESPONSE, CARDIOVASCULAR FUNCTION, AND FECAL BILE ACID EXCRETION DURING A 28-DAY FEEDING PERIOD IN DOGS: COMPARISON WITH COMMERCIAL DIETS WITH NORMAL VS. HIGH PROTEIN

4.1 Preface

Chapter 4 is the longer-term study included in this thesis on fava bean fermentation as a pet food component and its effects on beagle health. Eight beagle dogs were examined in this research. This chapter explores the effects of fermentation of two varieties of fava beans on overall and cardiovascular health in dogs after 28 days of feeding, together with a comparison between two commercial pet food with different protein levels.

This chapter will be submitted for publication in the journal *Metabolites*. The author list is as follows: Luciana G. Reis (responsible for the study design, the fermentation process, the animal work and tests, the biochemical examinations excluding those sent to specific labs for analyses, the data analyses, and the writing), Tressa Morris (responsible for the fermentation process), Chloe Quilliam (helped with animal work), Lucas A. Rodrigues (responsible for the data statistics), Mathew E. Loewen (responsible for the study design and supplied with the fava bean flours), and Lynn P. Weber (supervised the studies, responsible for the study design, and the writing).

4.2 Abstract

We have recently shown that feeding high- or low-tannin fava bean-based diets for 7 days did not produce favism-associated toxicities in dogs. Furthermore, fermentation of fava bean flour with *Candida utilis* has the potential to further improve fava bean flour through decreased anti-nutritional factors. In the present study, the effects of 28-day feeding of 4 different fava bean-based test dog foods containing moderate protein (~27% dry matter (DM)) were compared with two

commercial diets with normal (NP, grain-containing, ~31% DM protein) or high protein (HP, grain-free, ~41% DM protein). Glucose tolerance, body weight, cardiovascular function, blood parameters, and fecal bile acid content were investigated in beagles fed the NP or HP diets or a randomized, crossover, 2×2 Latin square design of the fava bean diets: unfermented high-tannin (UF-HT), fermented high-tannin (FM-HT), unfermented low-tannin (UF-LT), and fermented low-tannin (FM-LT). As a result, fermentation increased glucose tolerance, increased red blood cell number, increased systolic blood pressure and improved cardiac elasticity indicated by decreased passive left ventricular filling (E wave), but decreased flow-mediated vasodilation. Taken together, the overall effect of fermentation appears to be beneficial and improved FB nutritional value. Most interesting, even though the HP diet was grain-free, several measures of cardiac function were improved, while glucose tolerance was impaired compared to NP-fed dogs. In summary, this study did not find evidence of adverse cardiac effects of pulses in these ‘grain-free’ diets, at least not in the relatively resistant beagle breed over a 28-day period. More importantly, fermentation with *C. utilis* shows promise to enhance health benefits of pulses such as FB in dog food.

4.3 Introduction

Fava bean (*Vicia faba* L.) is a proteinaceous ingredient with potential for partially replacing animal protein sources in human diets and has been associated with health improvements triggered by its functional properties (Sharan et al., 2021). In companion animals, despite the increased interest in incorporating fava beans in the diets, there are still concerns about potential toxicity from anti-nutritional factors (Rahate et al., 2021b), which prevent its approval as a feed ingredient for pet food by the American Association of Feed Control Officials (AAFCO). Moreover, complicating the approval of fava beans is a concern surrounding all pulses used in pet food. Specifically, the US Food and Drug Administration (FDA, 2019) reported that pulses may be linked to dilated cardiomyopathy (DCM) occurrence in dogs fed grain-free diets (i.e., food formulated with potatoes and pulse ingredients instead of grains). While the underlying cause and verification of this link remain unsolved, proof of safety for any pulses, particularly new ones to pet food such as fava beans is needed.

In dogs, DCM is one of the most prevalent, genetically-associated cardiac diseases and is most common in large or giant breeds (Dutton, 2018). One proposed mechanism by which nutrition may link to DCM is through taurine insufficiency (Fascetti et al., 2003b; Backus et al.,

2006b). Taurine is an important component of calcium reabsorption pathways within the sarcoplasmic reticulum and increases the sensitivity of the cardiac myofilaments to calcium (Bakker and Berg, 2002b). Thus, it is reasonable to infer that under suboptimal intake of taurine or its precursors (e.g., methionine, cystine), cardiac contraction could be compromised which in turn increases risk of developing DCM (Mansilla et al., 2019b). Since grain-free and pulse containing diets tend to be high in fiber, these diets may have either or both decreased protein digestibility with a decreased bioavailability of sulfur-containing amino acids or higher fecal excretion of taurine via taurocholate (Kim et al., 1996c; Kim et al., 1996d). Plant proteins are naturally low in taurine precursor amino acids and completely lack taurine, compounding the taurine insufficiency issue.

There are several anti-nutritional factors in fava beans including condensed tannins, trypsin inhibitor activity, lectins, and pyrimidine glucosides that impair nutrient digestibility (Liener, 1989; Rahate et al., 2021b). Importantly, consumption of uncooked fava beans has been associated with sudden red blood cell death and acute anemia in humans with favism, a genetic mutation of glucose-6-phosphate-dehydrogenase (G6PD), that increases sensitivity to pyrimidine glucosides (vicine and convicine) toxicity. Fava bean fermented with *Lactobacillus plantarum* showed reduced vicine and convicine concentration with greater availability of free amino acids and protein digestibility (Santana and Empis, 2001; Coda et al., 2010; Curiel et al., 2015; Coda et al., 2015b). Furthermore, fermentation using the yeast *Candida utilis* may bring the dual benefit of reducing anti-nutritional factors and increasing taurine content (Hébert et al., 2013b).

We recently reported that fava bean-based diets did not cause hemolytic anemia and did not alter glucose response in dogs after a 7-day feeding period (Reis et al., 2021). Interestingly, fermentation with *Candida utilis* reduced the concentration of anti-nutritional factors and improved nutrient digestibility and red blood cell counts. Furthermore, fava bean-based diets were contrasted with commercial diets with normal or high protein, with the former showing possible detrimental effects on blood chemistry and improved cardiovascular functioning of dogs. Since the negative effects observed in our previous study could impact cardiovascular health to a greater extent if diets were fed longer than 7 days, studies using longer feeding periods are needed to assess safety.

Therefore, the objective of this study was to determine the effect of longer-term (28-day) feeding of beagles with unfermented or fermented fava bean-based diets on glucose response, body weight, cardiovascular function, blood parameters, and fecal bile acid content when contrasted to commercial diets with normal vs. high protein. It was hypothesized that pulse-based diets would impair cardiovascular health and increase blood markers of cardiac damage (cardiac troponins, pro-B-type natriuretic peptide or pro-BNP) due to the low taurine, cysteine, or methionine levels and high fiber content. Moreover, fermentation of fava bean flour with *Candida utilis* would enhance diet quality and, consequently, health of dogs.

4.4 Materials and Methods

4.4.1 Fava bean ingredients and fermentation protocol

Dehulled LT (Snowdrop) and HT (Florent) fava bean varieties, genotypes cultivated in Saskatchewan (Fleury et al., 2015) were ground into flour via a 400- μ m screen. The methodology to ferment each type of fava bean was adapted from a process used, previously used in our lab, in pea flour (Curso Almeida, 2020). Concisely, *C. utilis* (ATCC 9950) was preserved in sterile 80% (v/v) glycerol solution at -80°C , before being reactivated in YGC agar plates when required (Yeast Extract Glucose Chloramphenicol Agar; catalogue number 95765; Sigma Aldrich, St Louis, MO). Incubation of seeded plates occurred at 30°C for 72 h. A flame-sterilized platinum needle was used to transfer two loops of colonies to a 250-ml sterile conical flask containing 100ml of YPD liquid medium (Yeast Peptone Dextrose—A1374501; ThermoFisher, Waltham, MA). For 12 to 15 hours the flask was incubated in a horizontal shaker incubator (30°C) at 120 rpm. The next step was to transfer 10ml of the cultured yeast mass into a 500-ml sterile conical flask containing 250 ml of YPD liquid medium. Following, the medium with the yeast was incubated on a horizontal shaker (30°C) at 120 rpm for an additional 12 to 15 h. A total amount of 20 kilograms batches of each fava bean genotype were blended with the yeast broth, ammonia, and sterile water to form a soft dough, then the fermentation occurred in an adapted cement mixer with the temperature maintained at 30°C . The fermentation slurry was mixed for 3min every hour for 72 h. To prove yeast viability and count throughout the process, samples were collected every 24 h, and serial dilutions incubations at 30°C for 72 h of mixture plated on sterile agar plates were performed. The fermented fava bean flour was then dried in an oven at 60°C for 48 h at WCVF prior to transport to the University of Saskatchewan Canadian Feed Research Centre (North Battleford, Canada) for grinding process of the fermented flours,

after that all test diets (both fermented and unfermented fava bean flours) were mixed, extruded, and vacuum coated with fat to create the final dry kibble format of the diets to be consumed in the feeding trials.

4.4.2 Animals and diets

The University of Saskatchewan Animal Care Committee (Animal Utilization Protocol #20190055) approved the animal use and procedures which adhere to the Canadian Council on Animal Care. In total, eight neutered beagles, four females and four males, at a mean age of 3.3 ± 0.9 years and, at ideal body weight (9.4 ± 2.4 kg), were acquired from King Fisher International (Toronto, ON, Canada) or Marshall Bioresources (North York, NY, USA) and accommodated at the Western College of Veterinary Medicine (Saskatoon, SK, Canada). The beagles were sheltered individually in 1.1×2.7 -m kennels with access to the outdoor through a kennel door at night and during feeding but kept in a group kennel area during the day. Every day for at least 1 h, volunteers walked or socialized with the dogs. To reduce the stress, all beagles were familiarized to procedures with rewards prior experiments start, so no anesthetics or sedatives were used in this study.

Six diets, in total, were tested. Low- or high-tannin fava bean flours, either fermented or unfermented, were used at 30% inclusion, with diet formulations indicated in Table 4.1. The concentration of fava-bean was chosen based on the knowledge that more than 30% could cause gastrointestinal issues due to the amount of antinutritional factors present in legumes seeds in general. However, there was a need to explore high amounts to investigate the possibility of issues like the reported in humans so high levels of inclusion would give us the possibility to detect any adverse effect from fava bean, in beagles. The four fava bean test diets were unfermented low-tannin [UF-LT; 27% crude protein (CP) DM], fermented low-tannin (FM-LT; 28% CP DM), unfermented high-tannin (UF-HT; 27% CP DM), and fermented high-tannin (FMHT; 28% CP DM). As a non-digestible marker, insoluble ash (Celite) was added at 1%. To achieve nutritionally balanced diets before extrusion under equal process conditions (Munsey, 1957), diets were formulated in agreement with the nutrient guidelines for adult dog maintenance set by AAFCO. The two extra diets tested were commercial diets containing normal-protein (NP; 31% DM CP) or high protein content (HP; 41% DM CP). Ingredient lists for the commercial diets are shown in Table 4.2 showing that the high protein diet had taurine added to it The

proximate analysis of all six diets were performed (Central Testing, Winnipeg, MB, Canada) after being randomly subsampled (Table 4.3). The analyzed levels of crude fiber, non-fiber carbohydrates, metabolizable energy, vicine, and convicine of UF-LT, UF-HT, FM-LT, and FM-HT diets are 1.1, 0.5, 0.5, and 0.5%; 60.7, 62.1, 61.6, and 61.8%; 3.7, 3.8, 3.8, and 3.8 kcal/g; 1.8, 1.8, 0.4, and 0.6 mg/g; and 0.5, 0.6, 0.1, and 0.2mg/g, respectively. For crude fiber, non-fiber carbohydrates, and metabolizable energy of NP and HP diets, the analyzed content are 0.8 and 1.1%, 53.0 and 29.8%, and 4.0 and 4.1 kcal/g, respectively. The feeding period of each diet occurred for 28 days, with the NP or HP diets being used on the first and sixth months, respectively. From months 2 to 5, using a randomized, crossover, 2×2 Latin square design, the UF-LT, FM-LT, UF-HT, and FM-HT diets were fed. The two commercial diets were not incorporated into the crossover design as the fava bean-based diets were formulated to include the same nutrient profile to enable a satisfactory comparison. On the other hand, NP and HP diets have different components at unidentified inclusion amounts (specific formulation not available on label), making direct comparisons problematic. All beagles were weighed at the start of the experiment and after each feeding period. Standard equations were used to determine the energy requirements for individual dog maintenance [maintenance energy (ME in kcal)] = $[(70 \times BW^{0.75}) \times 1.6]$, so the quantity of diet assigned per dog per day is isocaloric with the daily requirement for that dog, daily amounts were apportioned, and dogs were fed twice daily (at 08:30 and 16:30 h). Before the following meal, bowls were removed, and any food left uneaten was weighed and recorded. No palatability issues were observed (Morris, Reis and Weber, unpublished) as all dogs usually ate all food portioned in each meal within the period of 5-10 min.

Table 4.1 Diet formulation for lab-formulated fava bean diets (%).

Ingredient	Unfermented		Fermented	
	Low tannin	High tannin	Low tannin	High tannin
Wheat flour	53.28	53.20	53.28	53.20
Low tannin fava bean fermented	-	-	30.00	-
Low tannin fava bean unfermented	30.00	-	-	-
High tannin fava bean fermented	-	-	-	30.00
High tannin fava bean unfermented	-	30.00	-	-
Turkey meal	11.74	13.04	11.74	13.04
Canola oil	1.40	1.00	1.40	1.00
Celite TM	1.00	1.00	1.00	1.00
Vitamin mixture	1.00	1.00	1.00	1.00
Mineral mixture	0.10	0.10	0.10	0.10
Salt	0.30	0.30	0.30	0.30
Dicalcium phosphate	0.53	0.26	0.53	0.26
Calcium carbonate	0.55	-	0.55	-
Choline chloride	0.10	0.10	0.10	0.10

Table 4.2. Ingredient composition of normal and high protein commercial diets. Ingredients are listed in order of decreasing inclusion.

Normal protein commercial diet
Chicken, Brewers Rice, Whole Grain Wheat, Poultry By-Product Meal (Natural Source of Glucosamine), Corn Gluten Meal, Whole Grain Corn, Animal Fat Preserved with Mixed-Tocopherols (Form of Vitamin E), Corn Germ Meal, Fish Meal (Natural Source of Glucosamine), Animal Digest, Dried Egg Product, Salt, Potassium Chloride, Calcium Phosphate, Calcium Carbonate, Vitamin E Supplement, Choline Chloride, Zinc Sulfate, Ferrous Sulfate, L-Ascorbyl-2-Polyphosphate (Source of Vitamin C), L-Lysine Monohydrochloride, Manganese Sulfate, Niacin, Vitamin A Supplement, Calcium Pantothenate, Thiamine Mononitrate, Copper Sulfate, Riboflavin Supplement, Vitamin B-12 Supplement, Pyridoxine Hydrochloride, Garlic Oil, Folic Acid, Vitamin D-3 Supplement, Calcium Iodate, Biotin, Menadione Sodium Bisulfite Complex (Source of Vitamin K Activity), Sodium Selenite.
High protein commercial diet
Chicken Meal, Turkey Meal, Salmon Meal, De-Boned Chicken, De-Boned Turkey, De-Boned Trout, Potatoes, Chicken Fat (Preserved With Mixed Tocopherols), Peas, Tapioca, Lentils, Duck Meal, Chickpeas, Natural Chicken Flavor, Whole Dried Egg, Apples, Herring Meal, Flaxseed, Salmon Oil, Alfalfa, De-Boned Duck, De-Boned Salmon, Sweet Potatoes, Potassium Chloride, Pumpkin, Carrots, Bananas, Blueberries, Cranberries, Broccoli, Blackberries, Squash, Papayas, Pomegranate, Dried Chicory Root, Dried Lactobacillus Acidophilus Fermentation Product, Dried Enterococcus Faecium Fermentation Product, Dried Aspergillus Oryzae Fermentation Extract, Dried Bacillus Subtilis Fermentation Extract, Choline Chloride, Vitamins (Vitamin A Supplement, Vitamin D3 Supplement, Vitamin E Supplement, Niacin, L-Ascorbyl-2-Polyphosphate (A Source Of Vitamin C), D-Calcium Pantothenate, Thiamine Mononitrate, Beta-Carotene, Riboflavin, Pyridoxine Hydrochloride, Folic Acid, Biotin, Vitamin B12 Supplement), Minerals (Zinc Proteinate, Iron Proteinate, Copper Proteinate, Zinc Oxide, Manganese Proteinate, Copper Sulphate, Ferrous Sulphate, Calcium Iodate, Manganous Oxide, Selenium Yeast), Sodium Chloride, Taurine, Yucca Schidigera Extract, Dried Rosemary, Green Tea Extract, Peppermint, Parsley, Rosehips, Zedoary, Dandelion, Chamomile, Ginger, Fennel, Turmeric, Juniper Berries, Licorice, Marigold Extract, Cardamom, Cloves.

Table 4.3 Proximate composition (% , dry matter basis) of lab-formulated fava bean diets compared to commercial diets.

Item	Unfermented		Fermented		Commercial	
	Low Tannin	High Tannin	Low Tannin	High Tannin	Normal Protein	High Protein
Moisture	9.53	9.11	7.80	9.10	6.69	5.84
Dry matter	90.47	90.89	92.20	90.90	93.31	94.16
Crude protein	27.42	27.25	27.94	27.98	31.25	40.73
Crude fiber	1.05	0.47	0.50	0.52	2.32	1.05
Fat	2.71	2.51	2.07	2.15	13.45	17.38
Ash	7.20	6.74	7.01	6.67	6.84	10.07
Cystine	0.35	0.63	0.48	0.57	0.63	0.15
Methionine	0.32	0.29	0.31	0.30	0.50	0.61
Taurine	0.06	0.06	0.06	0.06	0.09	0.23
Non-fiber carbohydrates	60.72	62.12	61.56	61.77	45.21	29.83
Total digestible nutrients	82.85	83.88	83.57	83.90	81.91	79.94
Metabolizable energy (kcal/g)	3.72	3.80	3.76	3.80	4.12	4.09
Insoluble HMWDF* % (w/w)	3.90	3.00	4.00	3.60	5.00	4.60
Soluble HMWDF* % (w/w)	0.70	0.90	0.70	0.90	0.50	1.40
Total HMWDF* % (w/w)	4.60	3.90	4.70	4.50	5.50	6.00
Vicine (mg/g)	1.76	1.77	0.36	0.61	-	-
Convicine (mg/g)	0.50	0.58	0.12	0.22	-	-

*High Molar Weight Dietary Fiber

4.4.3 Digestibility protocol

Feces was collected for 2 subsequent days after a 26-day feeding period on each diet (total of 28 days on each diet). Feces were stored in containers and labeled, and frozen at -20°C until analysis. Feces samples were thawed, homogenized, and pooled by dog (i.e., two samples from each dog pooled per dietary treatment). Prior to laboratory testing, feces were dried in a forced air oven at 55°C for 72 h and ground in a cutting mill with a 1-mm sieve. Dry matter by oven-drying the sample, non-fiber carbohydrates, crude protein applying the Kjeldahl method, and acid-hydrolyzed fat were analyzed (Central Testing, Winnipeg, MB) from feces and diets, according to AOAC standards (Horwitz et al., 1970). A bomb calorimeter was used to determine the content of GE of diets. To calculate the apparent digestibility coefficients of dry matter, crude protein, non-fiber carbohydrates, fat, gross energy, methionine, cysteine, and taurine for each fava bean-based diet using Celite as a non-digestible marker (Zhang and Adeola, 2017), the following equation was used:

$$\text{Digestibility (\%)} = 100 - [100 \times (M_{\text{feed}} \times C_{\text{feces}} / M_{\text{feces}} \times C_{\text{feed}})]$$

Where C feed and C feces indicate amount of components of interest in feed and feces, respectively; M feed and M feces indicate concentration of index compound in feed and feces, respectively.

4.4.4 Oral glucose response test and blood analysis

A 10% glucose solution (1 g/kg BW glucose) was fed to each dog at the same time each day, after 28 days of feeding each diet and an overnight 8 h fasting, by placing a syringe with the solution in the back of the mouth, for the conduction of an oral glucose tolerance test. Previously to the glucose feeding, the fasted dogs were aseptically catheterized in the cephalic vein using a peripheral intravenous catheter equipped with an extension tube. Before feeding glucose (time 0) and at 30 min after feeding, as 30 min was previously observed as the glucose peak concentration (Reis et al., 2021), blood samples (~0.2ml) were collected. Blood glucose was measured using a glucometer (OneTouch Ultra 2; LifeScan, Johnson & Johnson, New Brunswick, NJ) with at least two readings for each time or until two consistent readings were achieved. To guarantee no contamination of blood with anticoagulant solution, the extension tube was loaded with blood and the initial blood was discarded prior the glucometer reading.

Afterwards, the catheter was flushed with a sterile citrate solution to prevent any clotting right after each blood sample was collected.

Extra fasting blood samples (8ml) were obtained using collection tubes with and without EDTA.

Complete blood cell count [red (RBC) and white (WBC) blood cell counts] and chemistry panel [cholesterol; total (TB), direct (DB), and indirect bilirubin (IB); alkaline phosphatase (ALP); alanine aminotransferase (ALT); creatine kinase (CK); gammaglutamyltransferase (GGT); glutamate dehydrogenase (GLDH); total protein, albumin (A), globulin (G), and A:G] were analyzed at Prairie Diagnostic Services (Saskatoon, SK). Also, 3-ml subsamples of EDTA-tube blood from fasted animals were centrifuged at 2,000 rpm for 10min for plasma collection. Plasma samples were maintained at -80°C until further analyses of methionine, cystine, and taurine content (UC Davis Amino Acid Lab, Davis, CA). Unlike our previous study (Reis et al., 2021), the cysteine level was not analyzed by UC Davis Amino Acid Lab due to high instability of the parent cysteine compound. Plasma amino acid concentrations were analyzed in an automated amino acid analyzer via cation exchange high-pressure liquid chromatography separation and ninhydrin-reactive colorimetric detection (Delaney et al., 2003a; Spitze et al., 2003b; Tôrres et al., 2003; Heinze et al., 2009).

4.4.5 Cardiac function, blood pressure, and vascular health

Dogs were examined for cardiovascular health after 28 days of feeding each diet. All ultrasound parameters were executed and analyzed by single sonographer who was a DVM with specific training in ultrasound technique and >200 hr echocardiography experience where repeatable measurements in the same beagles were determined prior to start of the current study. Blood pressure was obtained using a high-definition canine/feline oscillometer (VET HDO High Definition Oscillometer, Babenhausen, Germany), before ultrasound examination. An average of two readings with good agreement was utilized to establish diastolic and systolic pressures. To indicate vascular health, endpoints of flow-mediated dilation were used which included brachial artery diameter during baseline, during inflation of a blood pressure cuff placed distal to the brachial artery, and at the time of peak dilation (30 s) after cuff release, as established before by our research group in dogs (Raitakari and Celermajer, 2000; Adolphe et al., 2012; Reis et al., 2021). Echocardiography M-mode endpoints were used to assess cardiac function included heart rate (HR), stroke volume (SV), and cardiac output (CO) (Adolphe et al., 2012; Otto et al., 2019).

Additionally, left ventricular end-diastolic volume (EDV), left ventricular end-systolic volume (ESV), ejection fraction (EF), left ventricular diastolic free wall thickness (DWT), left ventricle systolic free wall thickness (SWT), systolic blood pressure (SBP), diastolic blood pressure (DBP), and maximum velocity of blood flow through the mitral valve (MV) were also quantitated. Flow-mediated dilation and echocardiography were measured using a SonoSite Edge II ultrasound (Fujifilm SonoSite, Bothell, WA) with detection using a P10x transducer (8–4Hz) to detect cardiac endpoints and the L38xi (10–5Hz) transducer for vascular imaging. The following equation was used to calculate the flow-mediated:

$$\%FMD = 100\% \times [(maximum\ diameter\ postcuff\ release) - (baseline\ diameter)] / (baseline\ diameter).$$

Two-dimensional guided M-mode echocardiography was used to obtain a right parasternal short-axis view of the heart at the level of the papillary muscles (Lang et al., 2005). Measurements of left ventricular end diastolic inner diameter (LVID_d) and left ventricular end-systolic inner diameter (LVID_s) were also compiled from all dogs and normalized to body weight according to methodology described by Cornell et al. (2004). Finally, the pro-B-type natriuretic peptide (proBNP, Biosite, San Diego, CA) and cardiac troponin (Canine hs-cTnI; Nordic Biosite, Täby, Sweden) contents were determined in plasma samples according to the manufacturer's instructions of the respective kits.

4.4.6 Fecal bile acid content

Bile acid levels in fecal samples were measured with Total Bile Acid Assay Kit (Cell-Biolabs, Inc., STA-631) according to manufacturer's instructions.

4.4.7 Statistical analysis

Analyses were executed using SAS (version 9.4; SAS Institute, Cary, NC). Prior to perform all analyses, the data were explored for normality and outliers using the PROC UNIVARIATE model in SAS and the Shapiro–Wilk test. NT-proBNP and Cardiac Troponin I data followed a non-parametric distribution and data was logged transformed to reach normality. One-way ANOVA was used to contrast differences among normal vs. high protein commercial diets and two-way ANOVA was used to contrast parameters for fava bean–based diets (fixed effects being fava bean variety and fermentation). In this current study, we used mixed-sex dogs

to control the variation possibly connected with this factor. Preceding studies from our group have not discovered sex-related differences among (spayed/neutered) dogs (Adolphe et al., 2012) and because sex-related differences are not associated to the rationale of the present study, we did not incorporate this factor in the model. All post-hoc analyses were executed using the Fisher least significant difference (LSD) method. Differences were considered significant at $P < 0.10$.

4.5 Results

4.5.1 Body weight, meal portion, and body condition score

Body weight (BW), BCS, and meal portion data are presented in Table 4.4. Body condition score was assessed using a 9-point scale (Laflamme, 1997). No significant effect ($P > 0.05$) of dietary protein content (NP vs. HP) was observed on BW, BCS or meal portion after 28 days of feeding in beagles. Likewise, within fava bean–based diets, there was no effect of either FM, FB, or the interaction between FM and FB on BW, BCS, or meal portion ($P > 0.05$).

Table 4.4. Body weight, food portion, and body condition score (BCS) of dogs fed diets formulated with either low or high tannin fava beans without (UF) or with (FM) fermentation, or normal (NP) vs high (HP) protein commercial diets for one month each*.

Item	Commercial			Low tannin		High tannin		SEM	P value [‡]		
	NP	HP	SEM	UF	FM	UF	FM		P	FB	FM
Body weight (kg)	9.40	9.20	0.88	9.20	9.19	9.15	9.28	0.84	0.87	0.98	0.94
Food portion (g/d)	89.75	84.75	8.16	79.57	99.50	96.43	81.38	11.01	0.67	0.96	0.83
BCS	4.50	4.75	0.22	4.88	4.63	4.63	4.88	0.26	0.43	1.00	1.00

*Eight mixed-gender, neutered beagles were fed the NP or HP diets during the first and sixth months, respectively. From months 2 to 5, using a randomized, crossover, 2×2 Latin square design, 4 diets differing in fava bean variety and fermentation were compared as follows: unfermented (UF) high tannin, fermented (FM) high tannin, UF low tannin and FM low tannin. Values expressed as means ($n = 8$). SEM = Pooled standard error of the mean.

[‡] P = P-value between commercial diets with different protein content (NP vs HP), FB = Fava bean (Low tannin vs High tannin), FM = Fermentation (UF vs FM). The interaction between FB and FM was not significant for any of the parameters measured ($P > 0.10$).

4.5.2 Glucose response

Blood glucose responses to the oral glucose tolerance test are shown in Figure 4.1. Baseline (0 min) blood glucose levels were not significantly different between NP and HP ($P > 0.05$) while HP-fed dogs showed greater glucose levels compared to NP at 30 min after glucose feeding ($P < 0.05$). Also, there was no effect of fava bean variety or fermentation at 0 min, while dogs fed FM-HT had lower glucose levels at 30 min after glucose challenge compared to UF-HT, with UF-LT and FM-LT being intermediate ($P < 0.05$).

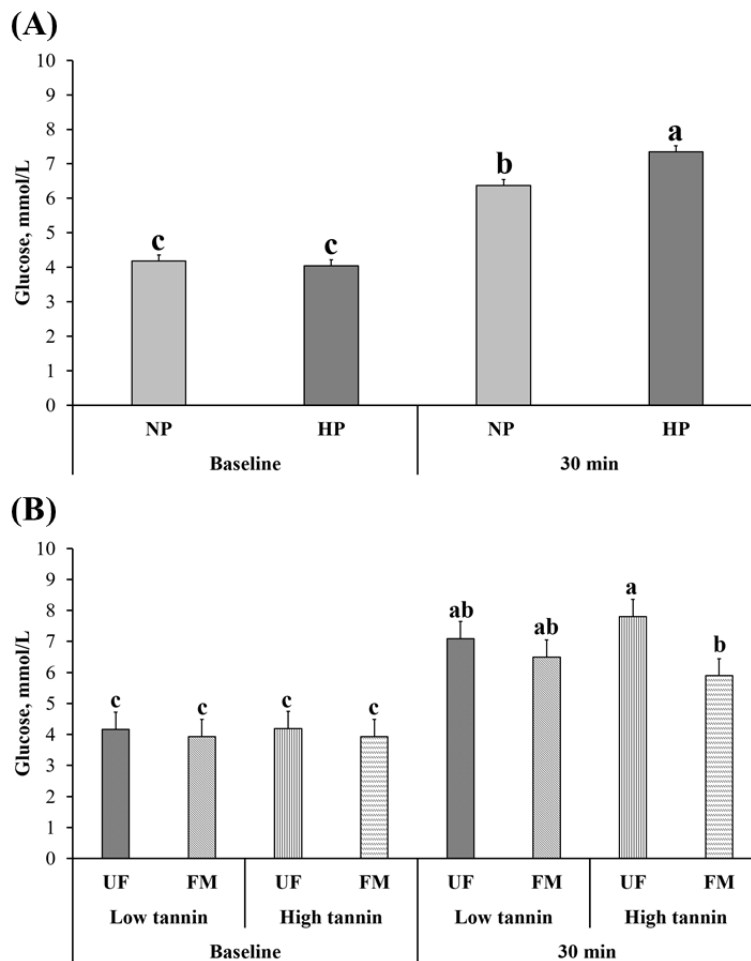


Figure 4.1 Oral glucose tolerance test in dogs fed either normal (NP) vs high protein (HP) commercial diets (A), or low vs high tannin fava beans-based diets without (UF) or with (FM) fermentation (B). Eight mixed-gender, neutered beagles were fed the NP or HP diets during the first and sixth months, respectively. From months 2 to 5, using a randomized, crossover, 2×2 Latin square design, 4 diets differing in fava bean variety and fermentation were compared as follows: Low tannin-UF, Low tannin-FM, High tannin-UF, High tannin-FM. Glucose levels correspond to baseline (0 min) and 30 min post feeding glucose solution. a–c Bars with different lowercase letters differ ($P \leq 0.05$).

4.5.3 Red and white blood cell count

Red (RBC) and white blood cell (WBC) counts of beagles after feeding each diet for 28 days are shown in Figure 4.2. There was no effect of dietary protein content on WBC or RBC levels ($P > 0.05$). Within fava bean-based diets, FM-LT dogs had greater RBC compared to UF-LT, with UF-HT and FM-HT being intermediate ($P < 0.05$). White blood cell count decreased ($P < 0.10$) in dogs fed FM compared to UF diets regardless of fava bean variety.

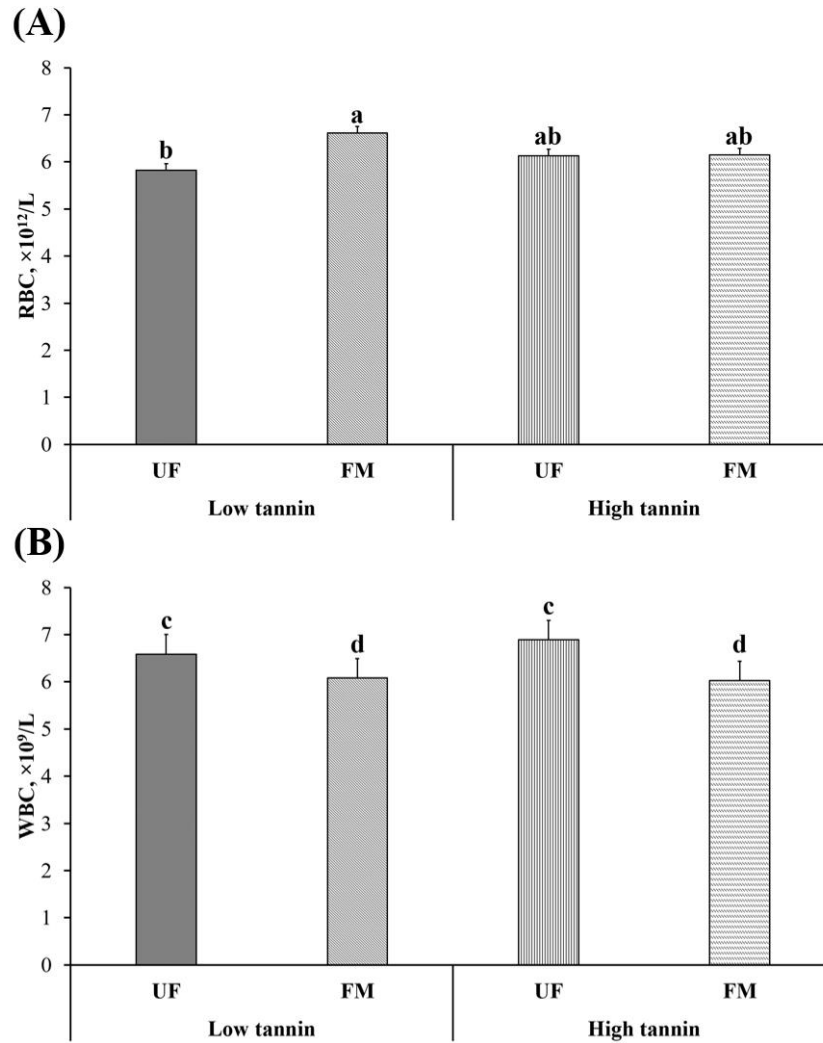


Figure 4.2. Red (RBC; A) and white (WBC; B) blood cell counts in dogs fed low vs high tannin fava beans-based diets without (UF) or with (FM) fermentation. From months 2 to 5, using a randomized, crossover, 2×2 Latin square design, eight mixed-gender, neutered beagles were fed 4 diets differing in fava bean variety and fermentation as follows: Low tannin-UF, Low tannin-FM, High tannin-UF, High tannin-FM. a–b Bars with different lowercase letters ($P \leq 0.05$). c–d Bars with different lowercase letters ($P \leq 0.10$).

4.5.4 Blood parameters of hepatic function

Blood parameters indicative of hepatic function in beagles after feeding test diets for 28 days are shown in Table 4.5. Within commercial diets, indirect bilirubin was lower while urea was greater in HP-fed dogs compared to NP ($P < 0.05$). Moreover, glutamate dehydrogenase decreased in NP compared to HP dogs ($P < 0.10$). Within fava bean-based diets, indirect bilirubin increased in HT compared to LT dogs regardless of fermentation ($P < 0.10$). Furthermore, gamma-glutamyl transferase decreased while alanine aminotransferase increased in FM compared to UF dogs regardless of fava bean variety ($P < 0.10$). There was an interaction between fava bean variety and fermentation where direct bilirubin was decreased in FM-HT compared to UF-HT dogs with UF-LT and FM-LT being intermediate ($P < 0.05$). No effect of dietary protein content, fava bean variety or fermentation were observed on cholesterol, total bilirubin, alkaline phosphatase, creatine kinase, total protein, albumin, globulin, albumin:globulin or creatinine levels ($P > 0.05$).

Table 4.5. Blood indicators of hepatic function in dogs fed diets formulated with either low or high tannin fava beans without (UF) or with (FM) fermentation, or normal (NP) vs high (HP) protein commercial diets for one month each*.

Item	Reference	Commercial		SEM	Low tannin		High tannin		SEM	P value [‡]			
		NP	HP		UF	FM	UF	FM		P	FB	FM	FB*FM
Cholesterol (mmol/L)	2.70-5.94	4.98	4.54	0.30	3.37	3.72	3.44	3.65	0.27	0.32	0.99	0.29	0.80
TB (μmol/L)	1.0-4.0	1.14	1.05	0.11	1.61	1.35	1.64	1.89	0.36	0.58	0.42	0.97	0.47
DB (μmol/L)	0.0-2.0	0.46	0.58	0.06	0.61 ^{ab}	0.63 ^{ab}	1.01 ^a	0.51 ^b	0.04	0.17	0.85	0.11	0.04
IB (μmol/L)	0.0-2.5	0.77	0.48	0.08	0.66	0.73	1.06	1.01	0.19	0.02	0.07	0.96	0.77
ALP (U/L)	9-90	37.63	30.57	4.87	76.63	65.50	64.75	52.86	10.73	0.34	0.24	0.26	0.97
GGT (U/L)	0-8	1.25	1.63	0.46	2.75	2.14	2.85	1.57	0.54	0.57	0.66	0.08	0.52
ALT (U/L)	19-59	20.00	19.50	1.34	20.43	25.38	24.50	26.25	1.97	0.80	0.23	0.09	0.43
GLDH (U/L)	0-7	2.43	3.38	0.36	3.14	2.63	3.13	2.86	0.30	0.07	0.71	0.19	0.67
CK (U/L)	51-418	152.75	146.43	20.53	142.86	182.25	160.12	171.71	29.03	0.82	0.90	0.37	0.63
Total protein (g/L)	55-71	50.63	47.57	4.91	54.50	55.13	54.38	54.63	1.33	0.65	0.81	0.74	0.88
Albumin (A; g/L)	32-42	32.00	34.50	1.39	33.25	34.00	33.75	33.63	1.17	0.23	0.95	0.79	0.71
Globulin (G; g/L)	20-34	18.63	18.63	0.72	21.25	21.13	20.38	21.00	0.77	0.99	0.52	0.74	0.63
A:G	1.06-1.82	1.74	1.88	0.12	1.59	1.63	1.65	1.61	0.09	0.42	0.78	0.97	0.64
Urea (mmol/L)	3.5-11.4	5.56	7.26	0.25	5.88	5.83	6.14	5.61	0.37	0.02	0.36	0.78	0.66
Creatinine (mmol/L)	41.121	60.00	67.38	5.03	61.75	55.29	64.13	62.38	6.03	0.65	0.36	0.22	0.19

*Eight mixed-gender, neutered beagles were fed the NP or HP diets during the first and sixth months, respectively. From months 2 to 5, using a randomized, crossover, 2 × 2 Latin square design, 4 diets differing in fava bean variety and fermentation were compared as follows: unfermented (UF) high tannin, fermented (FM) high tannin, UF low tannin and FM low tannin. Values expressed as means (n = 8). SEM = Pooled standard error of the mean. TB = Total bilirubin, DB = Direct bilirubin, IB = Indirect bilirubin, ALP = Alkaline phosphatase, GGT = Gamma-glutamyl transferase, ALT = Alanine aminotransferase, GLDH = Glutamate dehydrogenase, CK = Creatine kinase.

[‡]P = P-value between commercial diets with different protein content (NP vs HP), FB = Fava bean (Low tannin vs High tannin), FM = Fermentation (UF vs FM).

^{a,b}Means within a same row with no common superscript differ significantly (P < 0.05).

4.5.5 Blood electrolytes and enzymes

Blood electrolytes from beagles after feeding each test diet for 28 days are shown in Table 4.6. Within commercial diets, Na, anion gap, and Mg were increased in HP dogs compared to NP ($P < 0.05$). Moreover, Cl decreased ($P < 0.05$) while P increased ($P < 0.10$) in HP-fed compared to NP-fed dogs. Within fava bean-based diets, FM-fed dogs have greater anion gap compared to UF-fed dogs regardless of fava bean variety ($P < 0.10$). Moreover, FM increased lipase levels compared to UF regardless of fava bean variety (Figure 4.3, $P < 0.05$). Finally, there was an interaction between fava bean variety and fermentation where UF-LT dogs had greater P than UF-HT dogs with FM-LT and FM-HT being intermediate ($P < 0.05$). No effect of dietary protein content, fava bean variety or fermentation were observed on K, Na:K, HCO_3^- or Ca levels ($P > 0.05$).

Table 4.6. Blood electrolytes (mmol/L) of dogs fed diets formulated with either low or high tannin fava beans without (UF) or with (FM) fermentation, or normal (NP) vs high (HP) protein commercial diets for one month each*.

Item	Reference	Commercial		SEM	Low tannin		High tannin		SEM	P value [‡]			
		NP	HP		UF	FM	UF	FM		P	FB	FM	FB*FM
Na	140-153	145.87	147.63	0.49	146.87	146.50	146.63	146.86	0.37	0.02	0.88	0.85	0.42
K	3.8-5.6	4.65	4.71	0.06	4.63	4.61	4.54	4.64	0.06	0.44	0.66	0.53	0.42
Na:K	28-38	31.25	31.38	0.37	31.63	31.75	32.25	31.75	0.41	0.81	0.45	0.65	0.45
Cl	105-120	114.00	111.38	0.42	109.75	94.43	109.50	109.25	6.73	<0.01	0.30	0.27	0.28
HCO ₃ ⁻	15-25	18.50	18.75	0.48	19.38	18.38	17.29	19.13	1.05	0.71	0.53	0.69	0.19
Anion gap	12-26	18.38	22.50	0.57	22.63	24.00	19.86	22.88	1.27	<0.01	0.14	0.09	0.53
Ca	1.91-3.03	2.42	2.90	0.35	2.48	2.49	2.46	2.49	0.03	0.33	0.77	0.38	0.84
P	0.63-2.41	1.14	1.24	0.04	1.43 ^a	1.28 ^{ab}	1.23 ^b	1.29 ^{ab}	0.05	0.08	0.08	0.43	0.05
Mg	0.70-1.16	0.74	0.80	0.02	1.14	1.45	1.00	1.28	0.41	0.01	0.70	0.48	0.97

*Eight mixed-gender, neutered beagles were fed the NP or HP diets during the first and sixth months, respectively. From months 2 to 5, using a randomized, crossover, 2 × 2 Latin square design, 4 diets differing in fava bean variety and fermentation were compared as follows: unfermented (UF) high tannin, fermented (FM) high tannin, UF low tannin and FM low tannin. Values expressed as means (n = 8). SEM = Standard error of the mean.

[‡]P = P-value between commercial diets with different protein content (NP vs HP), FB = Fava bean (Low tannin vs High tannin), FM = Fermentation (UF vs FM).

^{a,b}Means within a same row with no common superscript differ significantly (P < 0.05).

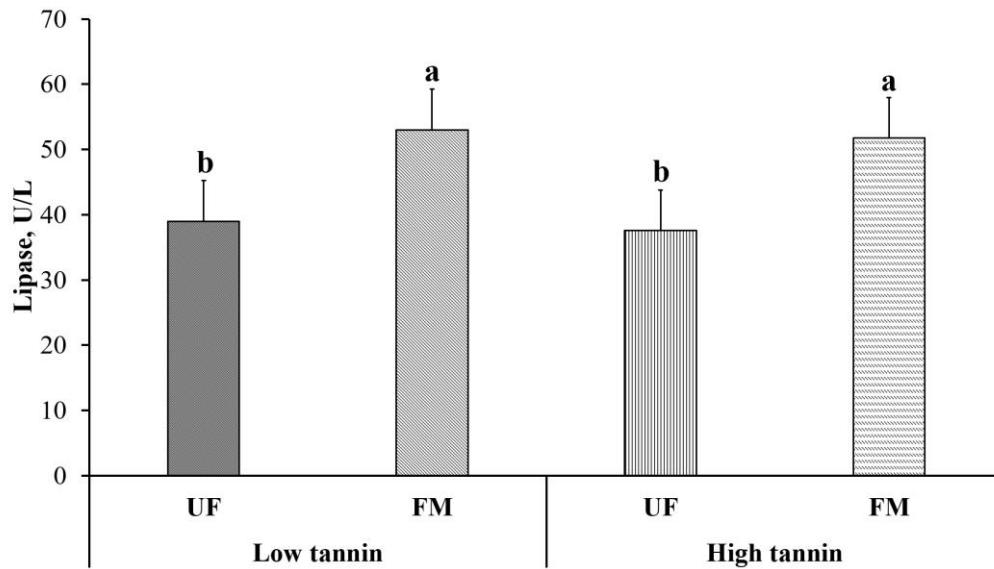


Figure 4.3. Blood lipase content in dogs fed either low or high tannin fava beans-based diets without (UF) or with (FM) fermentation. From months 2 to 5, using a randomized, crossover, 2×2 Latin square design, eight mixed-gender, neutered beagles were fed 4 diets differing in fava bean variety and fermentation as follows: Low tannin-UF, Low tannin-FM, High tannin-UF, High tannin-FM. a–b Bars with different lowercase letters differ ($p \leq 0.05$).

4.5.6 Cardiovascular function

Cardiovascular function parameters of beagles after 28 days of feeding each test diet are shown in Table 4.7. Feeding HP diets decreased left ventricular end systolic diameter and diastolic blood pressure, and increased ejection fraction (M-mode) and maximum velocity (A wave) while decreasing diastolic blood pressure compared to NP ($P < 0.05$). Moreover, dogs fed HP have greater left ventricular end-diastolic volume compared to NP dogs ($P < 0.10$). Within fava bean-based diets, HT-fed dogs had greater left ventricle diastolic wall thickness and lower maximum velocity (A wave) compared to LT regardless of fermentation ($P < 0.05$). Additionally, feeding FM diets increased diastolic blood pressure and decreased FMD compared to UF diets regardless of fava bean variety ($P < 0.05$). Moreover, left ventricular end-systolic volume increased in FM compared to UF regardless of fava bean variety ($P < 0.10$). Left ventricle systolic wall thickness was greater in UF-HT dogs compared to the other groups ($P < 0.05$). Also, systolic blood pressure was greater in FM-LT compared to UF-LT with UF-HT and FM-HT being intermediate. Finally, left ventricular end-diastolic diameter, heart rate, maximum

velocity (E wave), stroke volume (M-mode), cardiac output (M-mode), and E/A ratio were not altered by dietary protein content, fava bean variety or fermentation.

Regarding plasma levels of cardiac troponin I and NT-proBNP, no effect of dietary protein level, fava bean variety or fermentation were observed on those cardiac biomarkers.

Table 4.7. Cardiovascular function parameters of dogs fed diets formulated with either low or high tannin fava beans without (UF) or with (FM) fermentation, or normal (NP) vs high (HP) protein commercial diets for one month each*.

Item	Commercial			Low tannin		High tannin		SEM	P value [‡]			
	NP	HP	SEM	UF	FM	UF	FM		P	FB	FM	FB*FM
EDV M-mode (mL/kg BW)	1.72	2.39	0.25	1.99	1.98	1.21	1.95	0.25	0.09	0.72	0.59	0.62
ESV M-mode (mL/kg BW)	0.55	0.45	0.06	0.45	0.57	0.46	0.66	0.10	0.25	0.91	0.08	0.92
LVID _d (cm/BW ^{1/3})	1.17	1.24	0.04	1.17	1.15	1.14	1.16	0.04	0.27	0.82	0.95	0.67
LVID _s (cm/BW ^{1/3})	0.82	0.71	0.04	0.66	0.63	0.63	0.68	0.04	0.05	0.70	0.75	0.24
SV M-mode (mL/kg BW)	1.45	1.83	0.25	1.67	1.57	1.63	1.92	0.32	0.30	0.61	0.75	0.52
CO M-mode (L/min BW)	0.15	0.17	0.02	0.71	0.67	0.14	0.13	0.38	0.51	0.14	0.93	0.97
EF M-mode (%)	56.07	75.75	6.15	76.25	77.00	77.81	73.94	2.93	0.04	0.80	0.59	0.43
HR (bpm)	83.43	94.63	11.98	95.50	88.13	85.00	90.13	6.81	0.51	0.54	0.87	0.37
DWT (cm)	0.82	0.82	0.04	0.88	0.86	2.37	1.30	0.33	0.79	0.01	0.14	0.15
SWT (cm)	1.20	2.22	0.05	1.31 ^b	1.33 ^b	2.53 ^a	1.34 ^b	0.22	0.81	0.02	0.02	0.02
E/A ratio	0.18	0.19	0.01	0.15	0.16	0.18	0.16	0.24	0.63	0.69	0.68	0.65
MV (E wave) (cm/s)	71.61	69.31	12.07	80.53	69.16	67.93	77.20	10.55	0.90	0.83	0.92	0.33
MV (A wave) (cm/s)	23.15	37.03	4.31	40.39	36.46	31.49	29.59	3.65	0.05	0.04	0.45	0.79
SBP (mmHg)	136.40	127.25	7.13	117.19 ^b	152.79 ^a	132.31 ^{ab}	138.19 ^{ab}	10.00	0.38	0.97	0.16	0.05
DBP (mmHg)	75.73	60.50	3.95	67.19	81.96	66.31	75.58	6.02	0.01	0.55	0.05	0.65
FMD (%)	9.84	7.47	2.96	6.18	4.24	7.25	3.75	0.67	0.60	0.85	0.04	0.63
ProBNP (pg/mL)	15.08	8.84	8.28	23.93	15.38	7.44	17.87	9.07	0.60	0.45	0.92	0.31
Cardiac troponin I (pg/mL)	5.23	2.98	2.66	8.14	4.94	7.86	17.11	9.35	0.55	0.53	0.75	0.51

*Eight mixed-gender, neutered beagles were fed the NP or HP diets during the first and sixth months, respectively. From months 2 to 5, using a randomized, crossover, 2 × 2 Latin square design, 4 diets differing in fava bean variety and fermentation were compared as follows: unfermented (UF) high tannin, fermented (FM) high tannin, UF low tannin and FM low tannin. Values expressed as means (n = 8). SEM = Pooled standard error of the mean. EDV = Left Ventricular End-diastolic volume, ESV = Left Ventricular End-systolic volume; LVID_d = left ventricular end diastolic diameter, LVID_s = left ventricular end systolic diameter, SV = Stroke volume, HR = Heart rate, CO = Cardiac output, EF = Ejection fraction, DWT = Left ventricle diastolic wall thickness, SWT = Left ventricle systolic wall thickness, SBP = Systolic blood pressure, DBP = Diastolic blood pressure, FMD = Flow mediated dilation, E:A ratio: ratio between E and A wave MV (E wave) = Maximum velocity through mitral valve (E wave), MV (A wave) = Maximum velocity through mitral valve (A wave), ProBNP = pro B-type natriuretic peptide.

[‡]P = P-value between commercial diets with different protein content (NP vs HP), FB = Fava bean (Low tannin vs High tannin), FM = Fermentation (UF vs FM).

^{a,b}Means within a same row with no common superscript differ significantly (P < 0.05).

4.5.7 Digestibility

Apparent total tract digestibility in beagles fed fava bean–based diets are shown in Table 4.8. Dogs fed FM diets have greater non-fiber carbohydrates digestibility compared to UF diets regardless of fava bean variety ($P < 0.10$). Crude protein digestibility was greater in FM-LT compared to FM-HT with UF-LT and UF-HT being intermediate ($P < 0.05$). Moreover, gross energy digestibility was increased in FM-LT-fed dogs compared to FM-HT with UF-LT and UF-HT being intermediate ($P < 0.05$). No effect of dietary protein content, fava bean variety or fermentation were observed on fat, methionine, cysteine, or taurine digestibility.

Table 4.8. Apparent total tract digestibility of dogs fed diets formulated with either low or high tannin fava beans without (UF) or with (FM) fermentation for one month each*.

Item	Low tannin		High tannin		SEM	P value [‡]		
	UF	FM	UF	FM		FB	FM	FB*FM
Crude protein	85.38 ^{ab}	86.63 ^a	85.41 ^{ab}	84.20 ^b	0.43	0.07	0.98	0.04
Fat	76.79	75.94	88.12	83.94	8.35	0.24	0.75	0.83
Non-fiber carbohydrates	96.23	97.05	96.46	96.74	0.30	0.88	0.07	0.36
Gross energy	91.10 ^b	92.07 ^a	91.37 ^{ab}	90.88 ^b	0.32	0.15	0.46	0.03
Methionine	71.13	70.55	62.01	62.67	11.08	0.45	0.99	0.95
Cysteine	82.16	82.92	96.41	82.84	11.22	0.52	0.56	0.52
Taurine	81.32	91.85	91.00	90.35	6.51	0.51	0.43	0.37

*From months 2 to 5, using a randomized, crossover, 2×2 Latin square design, eight mixed-gender, neutered beagles were fed 4 diets differing in fava bean variety and fermentation as follows: unfermented (UF) high tannin, fermented (FM) high tannin, UF low tannin and FM low tannin. Values expressed as means ($n = 8$). SEM = Pooled standard error of the mean.

[‡] $P = P$ -value of FB = Fava bean (Low tannin vs High tannin) and FM = Fermentation (UF vs FM).

^{a,b}Means within a same row with no common superscript differ significantly ($P < 0.05$).

4.5.8 Plasma amino acid levels

Plasma amino acid concentrations in beagles after 28 days of feeding each test diet are shown in Table 4.9. Dogs fed HP diets had increased cystine concentration compared to NP-fed dogs ($P < 0.10$). Fermentation decreased cystine concentration compared to UF regardless of fava bean variety ($P < 0.05$). Beagles presented no significant differences in taurine and methionine plasma concentrations regardless of diet protein content, fava bean variety or fermentation.

Table 4.9 Plasma amino acid levels (nmol/mL) of dogs fed diets formulated with either low or high tannin fava beans without (UF) or with (FM) fermentation, or normal (NP) vs high (HP) protein commercial diets for one month each*.

Item	Commercial		SEM	Low tannin		High tannin		SEM	P value [‡]		
	NP	HP		UF	FM	UF	FM		P	FB	FM
Taurine	105.38	91.25	10.712	84.71	73.57	77.43	78.75	12.652	0.36	0.93	0.70
Cystine	16.29	22.13	2.287	20.00	17.50	19.00	15.13	1.502	0.09	0.27	0.04
Methionine	43.00	41.43	6.629	46.25	53.71	55.00	46.62	4.864	0.86	0.86	0.92

*Eight mixed-gender, neutered beagles were fed the NP or HP diets during the first and sixth months, respectively. From months 2 to 5, using a randomized, crossover, 2 × 2 Latin square design, 4 diets differing in fava bean variety and fermentation were compared as follows: unfermented (UF) high tannin, fermented (FM) high tannin, UF low tannin and FM low tannin. Values expressed as means (n = 8). SEM = Pooled standard error of the mean.

[‡]P = P-value between commercial diets with different protein content (NP vs HP), FB = Fava bean (Low tannin vs High tannin), FM = Fermentation (UF vs FM). The interaction between FB and FM was not significant for any of the parameters measured.

4.5.9 Fecal bile acid content

Fecal bile acid levels are shown in Figure 4.4. No effect of dietary protein content was observed ($P > 0.05$). Within fava bean-based diets, UF-LT and FM-HT dogs showed greater fecal bile acid levels compared to FM-LT with UF-HT being intermediate ($P < 0.05$).

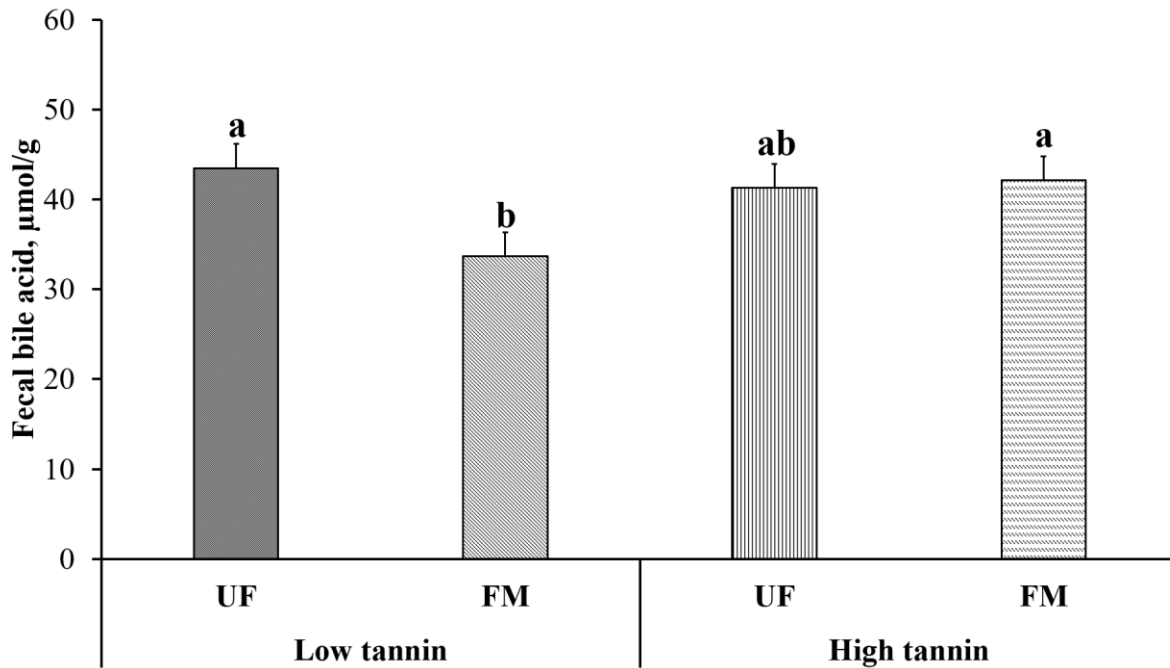


Figure 4.4. Fecal bile acid content in dogs fed either low or high tannin fava beans-based diets without (UF) or with (FM) fermentation. From months 2 to 5, using a randomized, crossover, 2×2 Latin square design, eight mixed-gender, neutered beagles were fed 4 diets differing in fava bean variety and fermentation as follows: Low tannin-UF, Low tannin-FM, High tannin-UF, High tannin-FM. a–b Bars with different lowercase letters differ ($P \leq 0.05$).

4.6 Discussion

We have previously reported that 7-day feeding of fava bean diets with moderate protein levels formulated to just meet the AAFCO requirements for methionine (0.33%) or methionine + cystine (0.65%) (AAFCO, 2017) did not produce clinical or physiological signs associated with cardiovascular impairment in adult beagle dogs (Reis et al., 2021). The most important finding in this current study was that dogs fed fava bean-based diet (30% inclusion) for 28 days also did not present impaired overall or cardiovascular health, with the plasma amino acids at safe and satisfactory levels. Furthermore, the high protein, grain-free commercial diet tested did not cause cardiovascular dysfunction when fed for the same 28-day period to dogs but instead showed slight improvements in cardiovascular functioning despite impaired glucose tolerance.

4.6.1 Toxicity from fava beans, digestibility, glucose tolerance, and anti-nutritional factors

In the present study, fermentation decreased the vicine and convicine content in fava bean flours and did not influence plasma taurine content or amino acid digestibility after 28-day feeding, which is in agreement with our previous findings in a short-term feeding period (Reis et al., 2021). It has been recently shown that fermentation with *L. plantarum* effectively degraded the pyrimidine glycosides in fava bean flour within 48 h, which attenuated the toxicity of the fermented diets (Rizzello et al., 2016b). Conversely, plasma cystine was decreased in dogs fed fermented diets and protein digestibility was greater in dogs fed fermented low tannin diets compared to fermented high tannin diets. This is contrary to our previous findings where protein digestibility was not influenced by fermentation, while plasma cysteine was increased in dogs fed fermented diets. A decreased plasma cystine content with the same taurine content in dogs fed fermented diets may indicate prioritization of endogenous cystine utilization for synthesis and normalization of plasma taurine concentration. Cystine is the predominant form of cysteine found in plasma and is a more stable, oxidized dimer of two cysteine molecules (Brigham et al., 1960). However, since we could not measure plasma cysteine due to chemical instability and technical issues, we cannot say with certainty whether total cysteine + cystine would have been changed in the same way as cystine alone. A greater protein digestibility in fermented low tannin compared to fermented high tannin may indicate that when feeding pulse-based diets for a longer period, the positive effects of fermentation may be dependent on tannin levels of fava beans prior to fermentation. This is an important finding of the present study since fermentation of

proteinaceous ingredients may benefit host overall health due to improvements in essential amino acids and bioactivity through digestion by the micro-organism (Ketnawa and Ogawa, 2019). Furthermore, fermentation increased plasma lipase content in the current study which was accompanied by a decreased fecal bile acid content in dogs fed fermented low tannin diets. These findings are somewhat non-specific and may not be linked, but could indicate an improved bile acid absorption in dogs fed fermented diets. Based on this effect, fermented diets have the potential to improve stool quality, since an excess of bile acids being spilled into the colon may stimulate electrolyte and water secretion, resulting in loose feces (Westergaard, 2007). Stool quality was qualitatively observed to be unchanged by diet in the current study, but at no time was feces dry or hard, agreeing with this hypothesis. This is also consistent with historical data in rats, where increasing fermentability of fibrous ingredients was directly associated with increasing bile acid excretion (Nyman et al., 1990). Finally, fermentation increased RBC content in LT-fed dogs only. Young dogs generally experience low RBC content due to RBC lifespan which is shorter and to the lower hemoglobin content of young RBC compared with aging RBC (Bush, 1991). An increased RBC content in low tannin fermented diet-fed dogs indicates altered fermentability between Snowdrop and Florent. This corroborates previous findings from our group and others of an accelerated production of blood cells and increased health (Çalışkantürk Karataş et al., 2017; Reis et al., 2021).

We used two fava bean varieties in the present study, which are genotypes commonly grown in Canada (Fleury et al., 2015), and generally classified as low-tannin (Snowdrop) (Wei, 2019) and high-tannin (Florent) (Fleury et al., 2015). However, the proximate analysis revealed high content of vicine and convicine in both fava bean varieties. High tannins could dramatically decrease nutrient bioavailability in the gastrointestinal tract (Bunglavan and Dutta, 2013; Addisu, 2016), particularly during an extended feeding period. We are unable to make inferences about bioavailability in the present study, however, fermentation was observed to increase protein digestibility in low tannin compared to high tannin fava bean varieties and increased gross energy digestibility in low tannin only. These findings contrast with our short-term study where there was no effect of fava bean variety on digestibility values of any nutrient measured (Reis et al., 2021). This indicates that the negative effects of anti-nutritional factors may be directly associated with feeding period length. Moreover, the ability of fermentation to improve apparent protein digestibility appears to be dependent on fava bean variety. One remarkable finding of the

present study was a decreased glucose response at 30 min after glucose feeding in dogs fed fermented high tannin fava bean-based diets compared to unfermented despite the lack of effect on fasting glucose. This suggests that long-term, fava bean varieties differentially influence glucose tolerance and/or that fermentation effects on glucose tolerance are only observed during a longer feeding period (Reis et al., 2021). It should be highlighted that the four-fava bean-based diets were fed sequentially in a crossover design for an overall period of four months, with no change in glucose tolerance from the previous NP period (first month). Furthermore, RBC content was within expected ranges in dogs fed fava bean-based compared to NP diets, which indicates that, even during a long-term feeding period, there was no anemia in the dogs and therefore favism was not a concern in the present study. Our long-term feeding trial provides additional evidence that fava beans should be further explored as an ingredient for dog food. However, a 6-month feeding study is required by AAFCO to approve a given ingredient to be used for pet food and this remains to be done.

4.6.2 High Dietary Protein Diet on Dog Health

In agreement with our recent study assessing a short-term feeding period (Reis et al., 2021), we confirmed that high dietary protein may negatively affect overall health of dogs, albeit to a lesser extent than previously observed. In the present study, urea was increased in HP compared to NP dogs. Higher urea signals kidney overload which could lead to increased proteinuria and potential impairment of the overall health of dogs (Burkholder et al., 2004). Furthermore, indirect bilirubin content was decreased in HP-fed dogs compared with NP, which is in line with Fisher et al. (2013) who reported higher bilirubin levels in young pigs fed a protein restricted diet compared with a control diet. In our short-term feeding trial (Reis et al., 2021), we observed potential positive effects of feeding HP diets to dogs including decreased ALP and ALT compared with levels of dogs fed NP which were not confirmed in the present study. Increased ALT content in dogs may be an indicator of hepatocyte membrane damage and necrosis, while increased ALP content may indicate biliary stasis (Willard and Twedt, 2012; Lawrence et al., 2018), which could be interpreted as poor hepatic function (Lucena et al., 2019). Since these observations were not replicated in a long-term feeding period and all values in this study were within clinical norms, the trends previously reported were, however, potentially random variation and are likely clinically insignificant.

4.6.3 Cardiac function, cystine, methionine, and taurine

The main objective of the present study was to feed pulse-based, high fiber diets to dogs aiming to investigate if the lack of effect on cardiac or vascular function observed after 7 days of feeding (Reis et al., 2021) would be sustained after 28 days of feeding. Specifically, changes in plasma taurine, cystine, or methionine levels, and/or alterations in cardiac contractility or enlargement of the heart consistent with DCM are of interest. Feeding high tannin fava bean-based diets increased left ventricle diastolic wall thickness and decreased maximum velocity of left ventricular filling during atrial contraction (A wave) compared to low tannin. This could indicate an elevated left ventricular mass (Rodrigues et al., 2017) which in conjunction with a decreased maximum velocity could be an initial signal of cardiac dysfunction. However, it should be noted that while statistically significant changes were observed in the current study, all values remained within clinical norms, indicating dogs remained healthy. Supporting the conclusion of cardiac health is the fact that neither proBNP or cardiac troponin levels changed with diet in the current study. These proteins, when present in the blood or plasma, are indicators of heart failure and cardiac damage, respectively (Oyama and Sisson, 2004; Ljungvall et al., 2010). Thus, our data do not support the hypothesis that fava bean-based dog foods cause cardiac impairment or DCM.

Surprisingly, diastolic blood pressure was increased, and flow mediated dilation was decreased in dogs fed fermented diets compared to unfermented regardless of fava bean variety, both of which would be considered adverse changes in vascular health. Flow mediated dilation, which is the vasodilatation of an artery following an increase in luminal blood flow and internal-wall shear stress, has been widely used as a surrogate, non-invasive marker of vascular health. The measurement of FMD is positively associated with endothelial nitric oxide synthase expression, and nitric oxide bioactivity (Berdeaux et al., 1994; Tuttle et al., 2001). As stated previously, feeding fermented diets produced a decreased FMD accompanied by an increase diastolic blood pressure. These effects are consistent with nitric oxide quenching due to increased oxidative stress which can be counteracted through dietary consumption of many plant polyphenols that act as antioxidants (Prior et al., 1998; Kähkönen et al., 1999; Rochfort and Panozzo, 2007). It appears that oxidative stress promotes cellular damage resulting in endothelial dysfunction and a gradual worsening of vascular health (Bielli et al., 2015). Fermentation may

have played a role in reducing the beneficial antioxidant effects while at the same time reducing the anti-nutritional factors in fava bean such as vicine/convicine. Considering that the animals were healthy, and no clinical or physiological effects were observed, losing the toxic compounds provides greater benefit than negative from a slight loss in antioxidant capacity.

On the other hand, the present study also provides indication of potential benefits of feeding the tested HP diet which were not observed at the short-term (Reis et al., 2021). Left ventricular end systolic diameter and diastolic blood pressure were decreased while ejection fraction and maximum velocity of active ventricular filling (A wave) were increased in HP compared to NP dogs. In humans, increased left ventricular end systolic diameter has been associated with mitral valve dysfunction (Tribouilloy et al., 2009) which could lead to diastolic hypertension (increased diastolic blood pressure) (Flint et al., 2019). Thus, the fact that the HP diet did the opposite and decreased these parameters is consistent with improved cardiovascular health. Conversely, decreased ejection fraction is a potent predictor of mortality and post-operative left ventricular dysfunction in human patients with chronic cardiovascular dysfunction (Bonow et al., 2006; Vahanian et al., 2007). In dogs, Adin et al (2019) showed that larger left ventricular end diastolic diameter, increased left ventricular end systolic diameter and lower sphericity index (left atrial to aortic ratio) indicate advanced myocardial dysfunction and remodeling in dogs. Moreover, left ventricular dilation and depressed systolic function, that can be assessed as reduced ejection fraction and decreased stroke volume and/or cardiac output, are abnormalities required to be present in order to diagnose dilated cardiomyopathy cases (Dukes-McEwan et al., 2003). However, none of those alterations were found with the dogs of the present study and in fact, ejection fraction improved, and again suggesting increased cardiac health with the HP diet compared to NP diet. Collectively, the present results may provide evidence for increased cardiovascular function in dogs fed this high protein, grain-free diet. This finding is significant since it does not support the statement previously made by the FDA (FDA, 2019) that many interpreted as all grain-free diets causing dilated cardiomyopathy in dogs.

The laboratory-formulated diets used in the present study had no significant differences in crude fiber content when compared to the commercial diets. Of note, cysteine content was decreased in fava bean-based diets compared to commercial diets. Moreover, methionine and taurine contents were higher in HP compared to either the NP diets or all fava bean-based diets.

An increased dietary taurine content or taurine supplementation is routinely used by veterinarians and nutritionists to improve cardiovascular functioning in dogs. Interestingly, our results from the current study's longer-term feeding period are in agreement with the that reported for a short-term feeding period e.g., 7 days (Reis et al., 2021), where plasma levels of taurine were not altered by diet and remained within the desired reference range in our beagles. Future studies should investigate whether more susceptible dog breeds (e.g., Doberman Pinscher, Great Dane, Boxer, and Cocker Spaniel) would show altered cardiovascular response when under the same dietary regimen.

Analyzed dietary methionine content was at AAFCO guideline levels or slightly suboptimal in NP and all fava bean-based diets. Sulfur-containing amino acids have a very dynamic metabolism, where dogs are able to synthesize taurine from cysteine or methionine (Harrison et al., 2020b). When plant-based protein sources such as pulses are fed, these amino acids are generally limiting due to lower levels in ingredients and /or high fiber content which decreases protein digestibility and increases fecal bile acid excretion (Pezzali et al., 2020b). The mechanism explaining effects of fecal bile acid excretion relies on high fiber content depleting taurine and decreasing protein digestion, which will further require cysteine and methionine to replace it. Contrary to our short-term study (Reis et al., 2021), fermented fava bean-based diets (both varieties) led to reduced plasma cystine levels after 28 days feeding each diet, although the cysteine measurements were the least reliable in this study due to instability. Despite this, changes in plasma cystine, taurine and methionine plasma levels were unaltered and levels remained above the reference range (Coda et al., 2010), possibly suggesting the cysteine results were spurious. Interestingly, fecal bile acid excretion was decreased in fermented, low tannin, fava bean-based diets. These findings combined suggest that reduced plasma cystine content could be associated with increased utilization for production of taurine in dogs fed fermented diets and that fermentation was able to attenuate fecal bile acid malabsorption (dependent on fava bean variety). Also in contrast to our short-term findings (Reis et al., 2021), there was no difference among commercial diets on cysteine, methionine, or taurine levels in the present study, which suggests that the alterations of blood levels of these amino acids observed after 7 days of feeding were not sustained at the long-term. Physiological compensation through adjustments in sulfur-containing amino acids metabolism or physiologic adjustments to maintain taurine balance may be responsible for lack of effect after longer term feeding.

4.8 Conclusion and Implications

One clear finding of the present study was that beagles fed the high protein commercial, or moderate protein, fava bean-based diets did not show any tendency toward developing DCM. Moreover, fava bean-based diets did not cause hemolytic anemia, both of which agree with our short-term trial in beagles. The period of one month does not provide enough time to draw conclusions related to long-term health. A period of 26 week is required to substantiate an adult maintenance claim for a dog food according to AAFCO regulations (AAFCO, 2017). Nonetheless, combined with the short-term study previously performed by the authors (Reis et al., 2020), these studies provide initial evidence to suggest FB could be included in dog food. However, feeding fava bean-based diets for the current one-month period did adversely alter glucose tolerance in a variety-dependent manner, but this was counteracted by fermentation. Specifically, fermentation with *C. utilis* effectively reduced anti-nutritional factors and improved overall health of dogs mainly through increased energy and nutrient digestibility as well as increased RBC levels. The previously reported results showing that a high-protein commercial diet impaired blood chemistry results compared with the normal protein were not sustained in the present study. Moreover, dogs fed the high protein diet (a grain-free diet) showed no evidence of adverse cardiovascular functioning compared to a grain-containing normal protein diet and if anything, showed small improvements. This diet did, however, vary in multiple ways from the other tested diets, such as inclusion of supplemental taurine, the use of multiple fruit and vegetable ingredients that could increase levels of beneficial phytonutrients such as polyphenols and antioxidants, as well as varying fiber sources and types. These contribution of these other variables remain to be explored.

CHAPTER 5

GENERAL DISCUSSION

5.1 Summary of conclusions

The main objectives of this thesis were to investigate the safety, health and cardiovascular effects and potential use of fava bean as a pet food ingredient. Also, study the effects of yeast fermentation with *C. utilis* of the fava bean-based diets and its impact on pulse anti-nutritional factors and, consequently, on dog health. In addition, two commercial diets with different protein levels, one grain-free and the other grain-containing, were combined to the research, and a comparison was made between them to observe health and cardiovascular outcomes on beagles. The first study analyzed a 7-day feeding period, and the second explored a longer feeding time: 28 days. Thus, a comparison over time between the two studies is possible. The results show that fava bean did not cause impaired cardiovascular functioning or induce anemia, even in the longer feeding period. So the overall hypothesis that a fava bean-based diet could cause cardiac impairments was rejected. Also, fermentation reduced important fava bean anti-nutritional factors and positively affected some health parameters, confirming the hypothesis that *C. utilis* fermentation would improve the diet's nutrient value.

The following is a summary of the principal findings of each study included in this thesis:

1. Fermentation with *Candida utilis* reduced the vicine/convicine content in fava bean flour on both varieties used.
2. After seven days, study 1 found that fava bean-based diets provoked no significant adverse cardiac or vascular function changes. In contrast, the NP commercial diet showed trends to impair systolic function in dogs.
3. Study 1 reported initial indications that fava bean is a safe ingredient in dog diets.

4. Study 1 concluded that glucose tolerance was not impaired on beagles fed fava bean-based diet, either the fermented or unfermented.
5. Study 1 showed that *C. utilis* fermentation on fava bean flour caused increased RBC levels and improved fat and carbohydrate digestibility.
6. Study 1 found that the HP commercial diet negatively impacted some blood health parameters in dogs, although the dogs were not clinically impaired and had positive effects on ALP and ALT.
7. Study 2 demonstrated that fermentation on low tannin fava bean variety caused a higher protein digestibility and increased plasma lipase content. In contrast, decreased fecal bile acid content in dogs fed fermented low tannin diets.
8. Study 2 determined increased RBC levels in low tannin fermented diet-fed dogs.
9. Study 2 found that fermentation increased protein and gross energy digestibility in low tannin variety.
10. Study 2 reported a decreased glucose response at 30 min after glucose feeding in dogs fed fermented high tannin fava bean-based diets.
11. Study 2 concluded that dogs did not have hemolytic anemia after 28 days of fava bean-based diets, which confirmed the safety of fava beans from study 1.
12. Study 2 concluded that fava bean-based diets did not cause cardiac impairment or DCM in dogs.
13. Study 2 showed that fermentation increased diastolic blood pressure and decreased flow mediated dilation.
14. Study 2 determined one particular HP, grain-free, diet was associated with beneficial cardiovascular changes.

The results of chapter 3 indicate that fava bean-based diets did not alter glucose handling nor caused acute hemolytic anemia in dogs after a 7-day feeding time, which points to fava bean being a safe ingredient for dog diets. Also, despite high fiber on the pulse-based diets, which was hypothesized to cause a decrease in taurine, cysteine, or methionine plasma levels and consequently heart dysfunction consistent with DCM, instead fava bean diets led to no

undesirable cardiovascular outcomes. In addition, fermentation is a promising bioprocessing technique to diminish anti-nutritional factors and potentially improve dog health by enhancing diet quality. On the other hand, the normal protein, grain-based, diet appeared to cause excess sympathetic tone, a trend that, if it were to continue with even longer term feeding than 28 days, might result in cardiac damage that is different from DCM. Thus, even longer-term studies are needed to verify if the lack of changes observed in this thesis with grain-free diets are sustained in dogs.

Chapter 4 outcomes show that fava bean-based diets or the HP commercial diet did not develop cardiac signs that could indicate DCM. Two cardiac biomarkers support the conclusion that no adverse cardiac alterations were caused. Also, in agreement with study 1, hemolytic anemia was not caused by fava bean-based diets. Nevertheless, the long-term fava bean feeding caused adverse glucose response in dogs fed the high tannin variety, which was stabilized by fermentation. The reduction of the anti-nutritional factors by the fermentation with *C. utilis*, specifically vicine and convicine, was confirmed. Despite showing a slight role in reducing antioxidant capacity from the fava bean, the benefits that fermentation brought were more valuable. Moreover, due to the increased energy and protein digestibility and improved RBC level, the net effect of fermentation is to enhance overall health in dogs. In addition, the negative effects of the HP commercial diet on blood chemistry results were not confirmed in the long-term feeding trial, suggesting physiological adaptation to the higher protein when fed for a period longer than 7 days. Interestingly, dogs fed HP, grain-free, diet seemed to result in improved cardiovascular functioning, which corresponds to the outcome from study 1. It is important observe that peas are the main pulse included in the HP commercial diet. Therefore the lack of cardiac impairment on dogs fed the HP or fava bean-based diets is of particular note since it provides evidence to refute the hypothesis put forward by the FDA that grain-free diets cause DCM in dogs.

5.2 Strengths and limitations

One strength of both studies was that we utilized two commercial brands to test against our lab-formulated fava bean-based diets, which gives a more representative scenario to the results. Another strength was the experimental design, employing a Latin square, crossover design where the same dogs were tested on each diet, diminishing variability and minimizing the issues associated with the reduced sample size of the experiments. Another strength of

this thesis was that we could compare results from study 1 to study 2 and put the outcomes together to infer how the feeding period affects the parameters analyzed. Also, we were the first to report fermenting fava beans with *C. utilis* which showed beneficial results in reducing vicine and convicine. Another strength was that we can corroborate the preexisting studies on fava bean for dog diet inclusion (Corsato Alvarenga and Aldrich, 2019; Corsato Alvarenga et al., 2020), showing that fava bean could be included in dog foods

The common limitation from both studies is that young and healthy adult beagles may show different responses than older, large breed dogs with a genetic predisposition to taurine deficiency. Moreover, due to the experimental design, which does not allow the statistical comparison of the commercial and fava bean-based diets, we are restricted to directly evaluating those two groups. Another limitation that is worth mentioning is that carnitine, an important amino acid to heart function or some of its precursors (e.g. lysine) were not assessed and thus we could not comment on any potential impacts for this study, but should be considered for future studies. Along the same lines, lysine could also have a vital role in maintaining cardiac health but was not investigated. Moreover, because fiber is such a complex nutrient, divided into many groups, not being able to study the diet levels and the role of each one of those in the obtained results impacted in the conclusions. Also, the commercial diets utilized have different ingredients and composition (the supplementation of taurine in the HP diet, for example) which imply that each diet could cause different effects on the tests made due to multiple variables not controlled for. Another limitation is that no control diet was used to compare the results to a baseline parameter.

5.3 Future work

The present studies provided evidence of the effectiveness of fermentation with *C. utilis* on reducing fava bean anti-nutritional factors, which is known to trigger favism in predisposed humans. This fermentation technique could be beneficial to prevent the development of the disease in humans, and research with rats as a model may bring new outcomes for susceptible subjects. Also, measuring the expression of G6PD enzyme and addressing the relationship with dietary interventions could indicate if a favism-like disease is possible in dogs.

Pulses, such as fava beans, are ingredients with low or limiting cysteine, taurine and methionine levels, as well as high fiber content. Although the lab-formulated fava bean-based

diets did not cause any significant decrease in plasma levels of taurine, cysteine, or methionine, a supplementation of crystalline sulfur amino acids in lab-formulated dog diets could cause different responses than the observed in our studies. Investigating carnitine and its precursors, such as lysine levels, in the studied diets or even their supplementation could make the study more broad and expand the discussion. Also, investigating the content of other fiber fraction (such as oligosaccharides) could improve the discussion in further studies.

Since DCM has many causes, it is important to acknowledge and investigate any other factor that could contribute to its development regardless of the nutritional aspect, such as potential unknown toxic effects of certain ingredients or nutrients. The ongoing investigations by various laboratories worldwide on how DCM could be linked to grain-free diets and this current study demonstrate that ingredients may not be indicated as the cause of a disease. The lack of effects of diets on cardiac changes in dogs fed with the fava bean-based diet or the high protein, grain-free diet implies that research on nutrient (instead of ingredient) composition of pet food is vital to achieve potential answers to that link. However, investigating the role of the ingredients present on the ingredient list of the diets could give us further idea on nutritive and antinutritive aspects of each ingredient and how they impact on dog's health. Future work regarding diets could be thought as making the diets more alike in terms of ingredient composition to investigate dog's overall and cardiovascular health.

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